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FUNCTIONAL SIGNIFICANCE OF VENOUS BLOOD PRESSURE

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THE SUPERFICIAL VEINS, by their appearance and by the pressures within them, have long been regarded as helpful indicators of the functional state of the more deeply situated, and less accessible, parts of the circulatory system. Black veins in the whites of the eyes suggested to Hippocrates a bad prognosis and for some conditions he recommended removal of blood by opening the inner vein of the arm. The venous valves and their relation to the filling of the veins in the forearm led Harvey to describe the circulation of the blood. Curate Hales measured venous as well as arterial pressures. Early clinical descriptions of patients referred to the distended, sometimes pulsating, peripheral veins associated with some diseases and the collapsed veins in conditions now recognized as shock, severe dehydration and peripheral vascular failure.

While Frank (1), Starling (2, 3) and Straub (4) studied the relation between venous return, auricular pressures and cardiac function in simplified circulations, Moritz and Tabora (5) refined the method of Hales and made direct determinations of peripheral venous pressure available at the bedside. Here nature's experiments, in the form of disease, add to the complexities of normal circulation the effects of a wide variety of primary and secondary disorders of the blood vessels, heart and lungs. The functional significance of venous blood pressure has interested both physiologists and clinicians as detailed reviews and monographs (6–14), together with innumerable briefer articles, indicate. The more recent insertion of catheters into peripheral veins (15) to measure pressures in the central veins and in the cavities of the heart (16) has been most helpful, particularly for studies in man whose vertical stance and whose diseases produce circulatory effects and compensatory adjustments not easily simulated by experiments in lower animals. Final interpretations are still frequently difficult, because venous blood flow and pressure depend to a large degree upon factors external to, or beyond, the walls of the veins themselves.

Figure 1 illustrates, much too diagrammatically, the quadrilateral interplay of the factors most important to venous pressure. They may be grouped into two general categories. First, the veins are situated between the capillaries (or their adjacent arteriovenous communications) and the heart. The blood enters the venules with appreciable *vis a tergo*, which remains after the capillaries have been traversed, and is then met by the *vis a fronte* which includes the resistance to flow on the part of the veins themselves and the resistance of the heart. Second, venous pressure is affected by internal factors, measurable as volumes or volume displacements, and by external factors, mural and extramural, generally measurable in terms of pressures.

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Beyond these directly acting factors, however, are more complex, and less direct, cycles by which a change of venous pressure produces physiological effects elsewhere and the latter then again tend to modify one or more of the direct forces and through them the original venous pressure. For example, venous pressure affects the relation between filtration and absorption of fluid through the capillary walls and, by this route, changes blood volume which, under some conditions, may again modify venous pressure. Also the 'effective blood volume' available to the heart and arterial vessels depends in part upon central venous pressure. Because the venous system is distensible, as well as contractile, blood may be 'pooled' in the peripheral vessels and veins in such volume that central venous pressure, cardiac output and arterial pressure fall despite compensatory vasoconstriction. Similarly, venous pressure both affects, and is affected by, cardiac output (1–4). The same relation holds for the described effects of venous pressure on cardiac rate (17), vasomotor tone (18), respiration (19, 20) and renal function (21a, 21b).

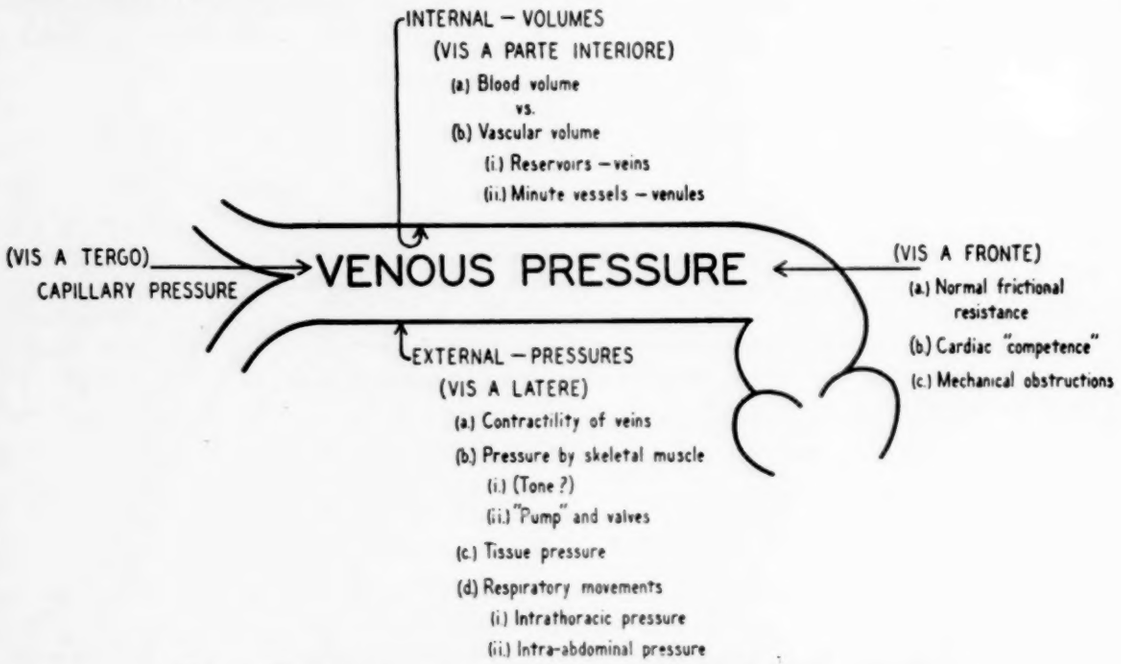

FIG. 1. FACTORS concerned in the maintenance of venous pressure.

Concerning the general functional importance of venous pressure, there can be little question. It is in the interpretation of the specific functional significance of normal, high or low venous pressures that difficulty arises. To avoid erroneous interpretations and to obtain maximum information concerning function from a pressure in which many interacting factors are concerned, it is particularly important to define the conditions under which determinations are made. In measuring venous pressure, interest is sometimes restricted to changes of local pressure; then the point of reference is often simply the level of the vein itself. More frequently peripheral venous pressures are referred to a suitable zero point to permit inferences concerning central venous pressures or right auricular pressures. The latter can also be measured directly by means of suitable venous catheters.

In this review the more directly acting factors (fig. 1) will not be described in great detail because this has been done quite amply in numerous earlier reviews (5–14). Emphasis will be placed on those studies providing quantitative or semi-quantitative information that may be useful in describing the functional significance of low and high venous pressures, the relations between peripheral and central venous pressures and the effects of rest and activity on these pressures.

VENOUS PRESSURE, FLUID FILTRATION AND BLOOD VOLUME

Under carefully standardized conditions of recumbency and rest, venous pressure of man and animals is relatively constant from moment to moment, except for slight

changes synchronous with respiration. This gives a highly limited view of the nature of venous pressure because conditions are customarily arranged to avoid disturbing variations. In activity such constancy cannot be expected because deeper breathing, muscular movements and changes of posture all affect venous pressure. Other factors remaining constant, every rise or fall will change capillary blood pressure and the balance between filtration and absorption of fluid through the walls of the capillaries. Since the total area of capillary endothelium in the body is great, and its permeability to fluid relatively high (22), considerable volumes of fluid can be filtered or absorbed in relatively short time if venous pressure rises or falls *1*) suddenly, *2*) in a large enough portion of the body and *3*) for a sufficiently long time.

Measurements of the relation between venous pressure and fluid movement in the human forearm by means of the pressure plethysmograph (23, 24) indicated that during inflation of a pneumatic cuff for periods of 10 minutes, fluid accumulated in the forearm tissues below the cuff at an average rate of 0.0033 cc/minute/100 cc. of forearm per centimeter water increase of venous pressure. A far more precise demonstration of the quantitative effects of venous pressure on filtration and absorption has been developed recently by Pappenheimer and Soto-Rivera (25) in the perfused, surviving hind limbs of cats and dogs. During perfusion with oxygenated blood, arterial and venous pressures could be changed separately or together at will, while net filtration or absorption was measured by recording graphically, and with great accuracy, any changes in the weight of the limb. In a limb previously isogravimetric, i.e. showing no change of weight because filtration and absorption were exactly equal, increasing venous pressure by 0.5 mm. Hg (0.7 cm. water) produced net filtration of fluid from the blood into the tissues of the limb at a readily detectable rate. Moreover, fluid movement was found to be 5 to 10 times more sensitive to changes of venous pressure than to changes of arterial pressure, i.e. similar increases of filtration rate were observed when venous pressure alone was raised by 5 mm. Hg and when arterial pressure alone was raised by 25 to 50 mm. Hg.

The filtration coefficients found in the cat and dog were respectively 0.012 and 0.014 gm/min/100 gm. of tissue/mm. Hg pressure difference across the capillary wall, corresponding to approximately 0.0095 per cm. water pressure difference. If it is assumed that elevating capillary pressure by 1 cm. water requires raising venous pressure by 1.25 cm. water, the effect of venous pressure on filtration becomes, for the cat and dog, approximately 0.0075 cc/min/100 gm. tissue/cm. water rise of venous pressure, i.e. over twice that found in the forearm of man. Pappenheimer and Soto-Rivera suggested (25) that the difference may be due to the greater capillary surface per unit volume of tissue in the dog's leg, when compared to the human forearm and cited in favor of this explanation the evidence that the reported values of blood flow per unit volume of tissue in the intact human forearm (26–28) are less than those in the hind limbs of anesthetized cats and dogs (29–31). It is also possible that the difference of filtration coefficients may be related in part to tissue pressure. In the perfusion experiments rates of fluid movement could be measured with very little change in the absolute volume of tissue fluid. Moreover, a given venous pressure usually continued to produce the same rate of filtration for several hours up to the time when massive edema appeared. In the intact human forearm (23, 24) and in

animal and human skin (32), on the contrary, calculated or measured interstitial pressures increased to as much as 35 cm. water during marked venous congestion. The filtration rates, which could not be measured accurately in man for periods of less than 10 minutes, declined slowly, despite constant venous pressure (23, 24). Hence, for man, the filtration rates so far obtained during congestion periods of 10 and 30 minutes represent something less than maximal filtrations and include the net effect of venous pressure and tissue pressure rather than the former only.

The filtration rates observed in the forearm seem inconsequential when expressed in unit terms but if calculated on the basis of a general rise of venous pressure throughout the body, i.e. a rise of auricular pressure transmitted throughout the whole capillary system, the total volume that can be filtered in a brief period is significant. According to the figure previously given (0.0033), a general rise of venous pressure to 10 cm. water above normal might, in a man weighing 75 kg., filter as much as 250 cc. of fluid from the plasma in the first 10 minutes. This calculation is based upon the rates of filtration observed in the forearm where the capillary walls are relatively impermeable to protein (33); if allowance were made for the considerably greater permeability of the hepatic and intestinal capillaries (34) the figure might be considerably greater. A higher local temperature would also increase these figures (24).

Total bodily filtration figures exceeding the estimate given above were obtained by an indirect type of experiment (35) in dogs, but such approximations cannot be applied directly to man because of species differences in filtration coefficients (25) as well as differences of blood reservoirs and of the musculature and reactions of the hepatic veins (36, 37). There is ample evidence, however, that when venous pressure in any considerable portion of the body is conspicuously elevated, blood volume decreases, e.g. in man by 11 to 15 per cent during prolonged quiet standing on a tilt table (38–40), and by even more in animals during prolonged centripetal acceleration in a centrifuge (41–43). In both situations venous pressure was elevated in only part of the body and in the centrifuge reached levels which were far beyond the physiological range. These figures show extreme effects but cannot be used to estimate the probable effects on blood volume of smaller and more generalized elevations of venous pressure.

A milder and more general venous congestion is found in the effects of pressure breathing which elevates venous pressure (44, 45) in accordance with the difference between the positive intrapulmonary pressure and the tension of the distended lung (46). Pressure breathing produced hemoconcentration corresponding to reductions of blood volume by 4 to 9 per cent in man, and 6 to 25 per cent in animals, the magnitude of change depending upon the pressure in the mask, the duration of pressure breathing, and the amount of external support (47, 48). In man this loss of blood volume, plus pooling of blood in the veins, probably exceeds 800 cc. and helps account for the syncope or collapse which is sometimes observed.

More recent studies (49) in which venous pressure has been measured directly during pressure breathing indicate that, over given periods, the total reduction of blood volume is proportional to the rise of venous pressure, just as local increase of the volume of tissue fluid in the forearm is proportional to the rise of venous pressure (23, 24). In periods of 20 minutes blood volume decreased approximately 2.5 per

cent for each 10-cm. rise in venous pressure. High environmental temperature increased this more generalized bodily filtration (49) just as heating the forearm increased local filtration (24) either because of increased filtering area or because arteriolar dilatation made capillary pressures higher at given venous pressures.

Repeated Valsalva maneuvers have also been used to increase venous pressure above the normal resting level by averages of 9 to 19 cm. water for periods of 30 minutes (50). During the first 10 minutes blood volumes were reduced by 94 to 449 cc. (average 240 cc.) with much less change, or even a slight tendency toward increase of blood volume, thereafter. The calculated bodily filtration rates observed during the first 10 minutes ranged from 0.0014 to 0.0034 cc/min/100-gm. body weight.cm. water rise of venous pressure. These figures, being based on hematocrits, are indirect; they may also fall short of true total bodily filtration because of the protective helmet and vest used in pressure breathing and contraction of abdominal muscles in the Valsalva maneuver. Nevertheless these figures are in accord with other evidence that a general elevation of venous pressure can, by increased filtration, reduce blood volume, rapidly and significantly during the first 10 to 20 minutes. Thereafter the effect on blood volume becomes much less, despite the probability that filtration is still continuing at a fairly rapid rate. This protection of blood volume from excessive change may be due to several factors. First, the net effect on blood volume will depend upon the promptness and rapidity with which the fluid filtered into the tissue spaces is returned to the blood stream via the lymphatics. Such adjustments are rapid and effective during muscular activity but slower and ineffective in quiescent tissues or in the presence of lymphatic obstruction and high lymph pressures (51). Second, it is likely that when venous pressure is elevated throughout the body, combined pooling of blood in the veins and filtration lead to compensatory vasoconstriction which may reduce filtration *1*) by reducing arteriolar capillary pressure toward the existing venous pressure, *2*) by diminishing the area of capillary wall available for filtration and *3*) by slowing or stopping blood flow in considerable sections of the capillary network. For maximal filtration, rapid renewal of capillary blood is required and this would not be the case in areas of marked vasoconstriction. Third, elevation of tissue pressure and increase of colloid osmotic pressure of the blood would also tend to lessen filtration over prolonged periods.

Closely related to the greater filtration of fluid produced by high venous pressure is the possible loss of plasma protein due to the stagnant hypoxia of venous congestion itself or due to mechanical distention of the capillary wall by increased internal pressure. By an earlier estimate, based on an indirect method, the capillary filtrate, produced in the human forearm by venous pressures of 40 and 60 mm. Hg, contained about 0.3 per cent protein, indicating that under these conditions the capillary endothelium of the extremities was approximately 95 per cent efficient as a protein-retaining membrane (33). Using a far more exact procedure and much lower venous pressures, Pappenheimer and Soto-Rivera (25) have reported similar results for the perfused hind legs of dogs and cats.

Complete cessation of blood flow increases the permeability of the capillary wall to protein apparently because of marked hypoxia (52, 53). In the human forearm

the oxygen saturation of venous blood must be reduced to the very low level of 20 per cent or less before the capillary filtrate contains an abnormal amount of protein (54). Similarly, very high venous pressures, 80 mm. Hg or more, are required to increase the passage of protein through the capillary wall (33, 43). The less marked elevations of venous pressure accompanying pressure breathing produced inconstant increase of protein passage (48). From data available it appears that venous pressures up to 60 mm. Hg increase the rate of filtration with little or no increase of protein concentration in the filtrate.

Within these limits the linear relation between venous pressure and the rate of filtration is, in fact, surprisingly good even though the veins and capillaries are separated by a potentially variable venular resistance. Capillary blood pressure must be exceedingly sensitive to changes in the ratio of venular to arteriolar resistance (25). If these two resistances should change in opposite directions, profound changes in capillary pressure and fluid movement would result. Studies of venomotor tone, as described below, indicate that generally the arterioles and venules tend to dilate or constrict together; under these conditions capillary pressure is unaffected. Though the actual occurrence of opposite reactions on the part of arterioles and venules has not been clearly demonstrated as yet, it is still conceivable that they may modify, in special situations, the slope of the curve relating filtration of fluid to venous pressure.

VENOUS PRESSURE, VASCULAR VOLUME AND 'EFFECTIVE BLOOD VOLUME'

Bayliss and Starling (55) observed that abolishing vasoconstrictor impulses by sectioning the cord, or the splanchnic nerves, reduced arterial pressure without changing venous pressure. This they explained by postulating that dilatation of the peripheral vessels *1*) lowers peripheral resistance, tends to increase *vis a tergo* and thereby venous pressure, but *2*) simultaneously increases the capacity of the whole vascular system, diminishes venous return and thus tends to decrease venous pressure. They estimated that section of the cord at the first dorsal vertebra increased the capacity of the vascular system of a 6-kg. dog by 125 cc., i.e. by a quarter of its blood volume.

Vis a Tergo. Venous blood pressure has its beginning in the *vis a tergo* which, as used in figure 1, refers to the residual pressure head remaining to the blood after it has passed through the major peripheral resistance situated in the arterioles and capillaries. The linear velocity of blood flow through the capillaries is so slow that the kinetic factor is negligible. In man by the time blood reaches the skin of the nail fold mean blood pressure has fallen from 100 mm. Hg in the aorta to 12 mm. Hg in the venous capillaries (56). In the mesentery of the rat and guinea pig the corresponding figures are 82 and 12 mm. Hg (57), and in the mesentery of the frog, 23 and 7 mm. Hg (58). Hence the transit of blood from the heart to the very beginning of the venous system dissipates from $\frac{7}{8}$ to $\frac{2}{3}$ of the pressure head supplied by the left ventricle, leaving available only $\frac{1}{3}$ to $\frac{1}{8}$ of this pressure to return the blood over the same distance to the right side of the heart. The larger fraction, $\frac{1}{3}$, applies to the mesenteric veins which are still separated from the vena cava by the resistance of the portal system and hepatic sinuses, while the smaller fraction, $\frac{1}{8}$, applies to the even longer vessels of the extremities connected directly to the heart by the large veins (59).

Even by casual observation the outstanding characteristics of the visible veins are their number and size. Quantitative data comprehensively comparing the cross-sectional areas of the arterial and venous systems are few and incomplete except for the mesenteric and pulmonary circulations (60). By taking the former as more generally representative, table 1 indicates that while the total cross-sectional area of the smallest venules at the base of the intestinal villi is only half that of the capillaries, it is still over twice the cross section of the corresponding arterioles of the villi. In the larger vessels the ratio is even higher, ranging from 4 to 6. This has direct bearing on the limited effects which *vis a tergo* exerts on venous pressure.

Maximal vasodilatation in the contracting muscles of the frog increased blood flow markedly and raised blood pressure most in the arteriolar capillaries, less in the venous capillaries, very slightly in the venules of the area and not at all in the nearby veins (61). In man reflex vasodilatation had no effect on venous pressure in the median basilic or saphenous veins (62). The diameter of the veins increased as the

TABLE 1. TOTAL CROSS-SECTIONAL AREAS OF MESENTERIC VESSELS OF DOG. CALCULATED AND RE-ARRANGED FROM SCHLEIER, (60)

ARTERIAL		VENOUS	
	*cm.*²	*cm.*²	
Mesenteric artery	0.07	0.28	Mesenteric vein
Main branches	0.12	0.67	Branches of mesenteric vein
End branches	0.13	0.79	Last branches of mesenteric vein
Short and long intestinal branches	0.20	0.84	Long and short intestinal veins
Last branches	0.57	2.32	Last branches of submucosa
Branches to villi	2.48	5.80	Veins before entering submucosa
Arterioles of villi	4.18	11.59	Venules at base of villi
		23.78	
		Capillaries of villi	

minute vessels of the finger tips dilated (62). In arteriovenous fistula venous pressure is high in the veins immediately connected to the fistulous tract, but central venous pressure remains low until cardiac failure supervenes (63–65). The widespread vasodilatation produced by amyl nitrite elevated venous pressure very briefly by 3.5 cm. water (66), and in reactive hyperemia (67) the rise of venous pressure was evanescent and small. The increased blood flow produced by clenching the fist 30 to 40 times in one minute did not elevate antecubital venous pressure by more than 1 cm. water in normal veins (68). The effects of increased *vis a tergo* on venous pressure are generally small owing to *1*) the large cross-sectional area of the veins and *2*) the venous dilatation that is usually associated with arteriolar dilatation (55, 62).

Conversely, under suitable conditions, vasoconstriction reduced venous pressure (62, 69) slightly, to approach the minimum level of 2 to 5 cm. water which persists even when the forearm is elevated above the heart level (70, 62). It may be concluded that in the recumbent subject, under normal conditions, large differences in *vis a tergo* produce at most small changes in venous pressure.

Under special conditions, however, when the flow of venous blood is impeded by organic obstruction or by gravity larger effects have been observed. In the pres-

ence of partial venous obstruction the increased blood flow of muscular contraction raised venous pressure distal to the obstruction to as much as 90 cm. water (68). In the upright position the sudden transition from walking to quiet standing produced very prompt filling of the leg veins and a very rapid rise of pressure within them when a warm environment had dilated the cutaneous vessels (49). In a cold environment, with vasoconstriction, venous filling was slower and the rise of venous pressure far more gradual as well as smaller in magnitude.

'Vascular Volume' and 'Effective Blood Volume.' The relation between the 'vascular volume' of a part and venous pressure is a reciprocal one in the sense that any rise of central venous pressure tends to fill and enlarge the veins as well as the venules and minute vessels. Conversely, any increase in the volume of the minute vessels, venules and peripheral veins reduces the rate of filling of the larger veins nearer the heart and tends to lower central venous pressure. Such volumetric shifts can be very large and, through changes of central venous pressure, can reduce the availability or circulating effectiveness of a given blood volume far more rapidly than a change of venous pressure can modify absolute blood volume by the filtration and absorption of fluid.

In this connection, terms such as 'effective blood volume' or 'actively circulating blood volume' are useful even though satisfactory methods of measuring these quantities have not yet been devised. Total blood volume may be normal, but active or passive increase in the volume of some or all of the peripheral vessels can reduce the volume of blood available to the heart per unit time for active recirculation. The final effects of gross maldistributions of this type are well known in the form of diminished arterial pressure and syncope. It is characteristic that the symptoms and signs of uncomplicated reductions of effective blood volume are most marked when the body is vertical. They are relieved, in most instances quite promptly, by placing the animal or subject in the recumbent position. As soon as the maldistribution is corrected, the existing blood volume again becomes 'effective.'

Such maldistributions have been observed during quiet standing on the tilt table (for review see 71, 72), quiet standing immediately after severe exercise (73–77), centripetal acceleration (for review see 78, 79), arterial puncture (80, 81), carotid sinus stimulation in some instances (82), and in spinal anesthesia (83, 84). The mechanisms responsible for the maldistribution are often complex, including combinations of venous pooling, arteriolar dilatation, filtration of fluid from the blood plasma, and acute tachycardia or bradycardia so that it is frequently impossible to designate any one factor as decisive.

The importance of the potential volume of the venules and veins is indicated, however, by the increased incidence of acute syncope produced during quiet standing, by nitrites despite compensatory vasoconstriction (85–87), by the effects of pathologically capacious veins (88), by the relief of symptoms by muscular movements (71, 72) or external support (74, 75, 79) and possibly by the improvement which physical training produces (72, 89). The vascular space involved in these reactions has characteristics which are neither simple nor completely studied. It may include arterioles, capillaries, venules and veins, or if compensatory vasoconstriction is present, it may include chiefly the venules and veins (85–87).

The volume-pressure curve at any moment will then be determined *1*) by simple filling at low pressures of flaccid, partially collapsed veins, *2*) at higher pressures, by stretching of the more or less elastic walls of veins and smaller vessels, *3*) affecting both the preceding, the tension exerted by the smooth muscle in the walls of veins, venules and minute vessels, and *4*) the support of surrounding tissues.

Table 2 presents data on the mesenteric vessels collected by Schleier (60) but now calculated in terms of volumes. True capillary volume, amounting to only 6 per cent of the whole mesenteric vascular volume, is small as it appears to be qualitatively in the living skin (90) and quantitatively in other tissues as well (91). Lewis (90) used the term "minute vessels" to include the terminal arterioles, capillaries and venules most directly concerned with local vascular reactions. Adopting this functional classification, rather than the histologic one, the collective vascular volume can be divided into minute vessels 19 per cent, large and small arteries 15 per cent, and large and small veins 66 per cent. For the pulmonary circulation these values were respectively 21, 26 and 53 per cent. Although these figures can hardly be accepted in any absolute sense for the living organism as a whole because they may have been affected by agonal vasoconstriction as well as the artefacts of fixation and sectioning, they provide an estimate of the potential capacity of the venous system. Moreover, recent volumetric studies with radioactive isotopes of iron and iodine (91) indicate that only 17 per cent of the total blood volume is within the minute vessels of the normal dog under light morphine narcosis.

Studies of vascular volumes that can be compared in terms of cc. of blood per 100 cc. of tissue during life are few because the ordinary plethysmograph measures merely changes of volume but does not permit estimating the basal volume of blood in the tissue prior to those changes and because the volume of the bloodless extremity is not known. This has been attempted in a few observations with the pressure plethysmograph (92) and also indirectly by means of differential blood volumes (93). The volume of blood in the uncongested forearm placed vertically at heart level (92) in a pressure plethysmograph at 34° C was, in one series, 2.7 cc/100 cc. of tissue. Venous congestions of 10, 20, 30, and 40 mm. Hg raised total vascular volume from the control level of 2.7 to 3.3, 4.3, 5.3 and 5.8 cc/100 cc. of tissue respectively. A venous pressure of 30 mm. Hg doubled the amount of blood in the tissues of the forearm.

The volume of blood in the legs has been measured (93) by using carbon monoxide to determine blood volume first while the circulation to the legs was obstructed by inflated pneumatic cuffs and then after the pressure in the cuffs was released. During rest with the legs horizontal, 12 to 15 per cent of the blood volume was in the legs, averaging in 2 subjects 4.4 and 6.6 cc/100 cc. of tissue, with considerable variation from day to day. This volume was greater than that found in the vertical forearm (92); the large veins of the latter were probably more nearly collapsed than those of the horizontally placed legs. Tilting for 3 to 5 minutes in the head down position (−60°) had little effect on the vascular volume of the legs in one subject whose visible veins appeared empty in the horizontal position but decreased the vascular volume of the other subject, whose leg veins seemed full in the horizontal position, from 6.6 to 4.6 cc/100 cc. of tissue. Conversely, tilting from horizontal to +60° (head up) had

less effect in the subject with full veins in the horizontal position (changed from 6.6 to 7.9 cc/100 cc. of tissue) and more effect in the subject with collapsed veins in the horizontal position (changed from 4.4 to 8.0 cc/100 cc. of tissue). While these 2 subjects had different volumes of blood in the legs in the horizontal position, they both had approximately 4.5 cc. of blood/100 cc. of leg tissue when the legs were elevated and nearly 8.0 cc. of blood/100 cc. of leg tissue when the legs were dependent.

TABLE 2. COLLECTIVE VOLUMES OF VESSELS COMPRISING THE MESENTERIC SYSTEM OF THE DOG (FROM SCHLEIER, 60)

NAMES OF VESSELS	NUMBER	RADIUS	LENGTH	VOLUME	PROPORTION OF TOTAL VOLUME	
		cm.	*cm.*	*cc.*		
Mesenteric artery	1	0.15	6.0	0.42	*Arteries and Arterioles* 20%	*Arteries* 15%
Main branches	15	0.05	4.5	0.55		
End branches	45	0.03	3.91	0.50		
Intestinal branches	1,900	0.0068	1.42	0.39		
Last branches	26,640	0.0025	0.11	0.06		
Branches to villi	328,500	0.00155	0.15	0.37		
Arterioles of villi	1,051,000	0.00112	0.20	0.83		
Capillaries	47,300,000	0.00040	0.04	0.95	*Capillaries* 6%	*Minute Vessels* 19%
Venules, base of villi	2,102,400	0.00132	0.10	1.15	*Veins and Venules* 74%	
Veins before submucosa	131,400	0.00375	0.10	0.58		*Veins* 66%
Last br., submucosa	18,000	0.0064	0.15	0.35		
Branches, short veins	28,800	0.0032	0.11	0.10		
Intestinal veins	1,900	0.0138	1.42	1.61		
Last branches	45	0.075	3.91	3.11		
Branches, mesenteric v.	15	0.12	4.5	3.05		
Mesenteric vein	1	0.3	6.0	1.70		
				15.72	100%	100%

During free standing, with postural sway, the volumes were 6.3 and 6.5 cc/100 cc. and during severe exercise, with an active 'muscle-pump' mechanism, ranged from 3.7 to 7.6 cc/100 cc. of tissue. These figures still do not indicate the full vascular volume that can be developed by maximal vasodilatation, because immediately after exercise, inactivity of the dependent legs permitted the accumulation in the lower extremities of 8.4 to 11.7 cc. of blood/100 cc. of tissue, corresponding to 25 per cent of total blood volume, or 12 to 15 per cent more of the blood volume than was present during rest in the horizontal position or even in active exercise. The muscles whose circulation could be blocked by a pneumatic cuff did not include all of the working muscles so that the total loss of effective blood volume was probably even greater. Displacements of similar magnitude were produced by venous congestion of 3 extremities (94); jugular venous pressure was reduced by 35 to 65 mm. water (95). The liver is probably capable of accommodating large volumes of blood with lesser

grades of congestion. In the cat hepatectomy reduced markedly the fall in arterial pressure produced by the head-up position (96).

Vasodilatation due to heat or sodium nitrite (85–87) increases vascular capacity at given pressures and lowers the venous pressure required to produce a given change of vascular volume. Capps (97) found that a congesting pressure of 20 mm. Hg produced 2 to 10 times as great an increment of limb volume at 42° C. as at 23°. Still larger increases were observed when reflex vasodilatation was produced in the hand under observation. At 42° the major change of venous volume occurred as venous pressure was elevated from 10 mm. to 20 mm. Hg, while at 23° it was necessary to increase the congesting pressure to 40 mm. Hg to produce a similar volume increase.

Having described the general magnitude of possible changes of vascular volume it is relevant at this point to consider certain physical properties of the venous wall and its surroundings, leaving venous contractility and 'venomotor tone' for a later section. Veins display conspicuous histologic differences (10, 98). They have been divided into categories on the basis of their muscular and elastic coats which are generally stronger in veins habitually exposed to high hydrostatic pressures and weaker in those occupying more sheltered positions, e.g. the upper extremity and head. Sphincter-like structures have been described in the veins of several organs (10). Hepatic sluice mechanisms (99) support the concept that blood may be stored in the liver, to be released to, or removed from, the general circulation as required (99–101).

Comprehensive comparative studies of the volume-pressure curves of excised veins are relatively few (98, 102–105) probably because it is recognized that, having sacrificed innervation and reduced the tension of their musculature, the results would demonstrate only partially the properties of the normal, living venous wall. It is generally agreed that for excised veins $\Delta V/\Delta P$ is very large until internal pressure reaches 5 to 10 cm. water and then decreases sharply, the elasticity being at high pressures almost the same as that of arteries. Veins are not all equally distensible (98, 105), and concerning changes of elasticity with age there is some doubt (98, 103).

Ryder, Molle and Ferris (105) divided the volume-pressure curves of veins *in vitro* into a first zone where 'free distensibility' of the collapsed vein produces a long vertical rise of the volume-pressure curve with little or no pressure change and, by fairly abrupt transition, the second zone of elastic stretch. The zone of free distensibility comprised from 50 to 70 per cent of the volume reached at higher pressures. The demonstration (105) that external pressure applied to the vein shifted the whole volume-pressure curve quantitatively is important for the relation between venous pressure and volume *in vivo*.

To what extent the veins in deeper tissues are within or beyond the free distensibility portion of their volume-pressure curve is uncertain. The superficial veins of the forearm are visibly collapsed when raised above the level of the clavicle. In this collapsed state venous pressure is approximately equivalent to tissue pressure (105, 106) and does not reliably reflect changes of auricular or central venous pressure. When the forearm is lowered below the clavicle the veins elevate the skin as they fill and as their cross-section changes from ellipsoidal to approach a circle. Plethysmographic measurements of distensibility of the venous system *in vivo* (103) have been

difficult to interpret because the initial degree of venous filling is unknown and because of inability to differentiate changes of venous volume from concomitant changes in tissue fluid volume and pressure. Similar difficulties were met in studying total distensibility of veins *in vivo* by measuring the small gush of blood leaving the femoral vein at zero pressure because the residual volume of the veins at this pressure could not be determined (107). The deep veins cannot be wholly collapsed even at low venous pressures during recumbency because the application of external pressure increased linear flow along the leg veins (108). Even though volume-pressure curves so far obtained *in vivo* are difficult to interpret in terms of free distensibility and of elasticity, it appears that during vasodilatation $\Delta V/\Delta P$ is much greater at lower, than at higher, venous pressures; vasoconstriction probably shifts the curve toward higher venous pressures and at the same time lowers its summit (85–87, 97). This suggests that the tension of the muscle fibers in the walls of veins and venules modifies the whole curve and that maximum distensibility may be found, depending on conditions, within the range of normal venous pressure or considerably above it. A new approach to this problem is needed before the combined effects of small changes of venous pressure and of venomotor tone on vascular volume can be formulated quantitatively.

The property of free distensibility and free collapsibility of veins allows external pressures—intrathoracic pressure, intra-abdominal pressure, the pressure of contracting skeletal muscle and the general tissue pressure—to assist or retard the return of venous blood to the heart. Effects of intrathoracic and intra-abdominal pressures are considered below in connection with cardiac filling. The well known muscle-pump mechanism, operating in conjunction with venous valves (for review see 109), needs no additional description nor evidence of its indispensable role in exercise except to note the efficacy with which it rhythmically reduces venous pressure in the legs (110–114) and keeps local vascular and blood volumes close to the normal resting level (93). The dependence of venous return and central venous pressure in exercise upon this mechanism is indicated also by the occurrence of syncope when quiet standing follows exercise (73–77).

During rest the effects of surrounding tissue pressure, or interstitial pressure, on venous pressure are less certain. Henderson (115–118) and more recently Gunther *et al.* (119–122) have attributed considerable importance to the effects of lowered tone of skeletal muscles and intramuscular pressure on venous pressure, particularly postoperatively, and in the circulatory failure of hemorrhage and shock, but this has been denied by others. Intramuscular pressure (123, 124) depends in part upon the intravascular and extravascular fluid which is present, and upon the tightness of the surrounding fascia. Venous congestion and muscular contraction elevated intramuscular pressures markedly in some muscles and much less in others. Venous congestion changed tissue pressure least in the easily distensible subcutaneous tissues (123–125). A great increase of extravascular fluid volume and presumably of tissue pressure maintained blood volume when the colloid osmotic pressure of the plasma had been reduced (126) but huge amounts of fluid, e.g. 0.5 to 1 l/kg. of body weight, were required. The intravenous injection of plasma produced a striking but delayed rise of intramuscular pressure (122). While tissue pressure must share in the external

support of the venules and veins, the varying relation between this pressure and the movement of fluid through the capillary wall makes it impossible to regard intramuscular pressure either as a valid measure of muscle tonus, or as dependent solely on muscle tonus. In subjects supported on a tilt-table, involuntary tensing of muscles and restlessness raised intramuscular pressure, apparently improved the return of venous blood to the heart and averted syncope (125), but this would seem to be merely an example of the muscle-pump mechanism. According to recent experimental tests, the tone of resting muscle plays at most a very minor role in maintaining the venous circulation (127–133).

VENOUS PRESSURE AND CARDIAC FUNCTION

Given normal *vis a tergo*, with normal vascular volumes and lateral pressures, the returning venous blood meets the resistance to flow offered by the veins and by the heart, which resistances collectively have been termed *vis a fronte* (fig. 1). The resistance of the veins is small whether estimated by calculation from the data of Schleier (60), table 2, or from direct measurements *in vivo* of the small pressure gradient that suffices for normal venous return in horizontally placed animals or man. However, attempts to relate peripheral venous pressure directly to cardiac function meet at once with difficulty *1*) because the thin-walled veins are freely collapsible and offer varying resistances to flow (105), *2*) because on entering the thorax they may be subject to angulation or compression by tensions from adjacent muscles or fascia and *3*) because the pressure within the thorax is below atmospheric level, and changes with respiration.

In dogs, reducing intrathoracic pressure by negative pressure breathing made right auricular pressure more negative (with respect to atmospheric pressure), but peripheral venous pressure did not fall by exactly corresponding amounts (134–137). The gradient of venous pressure increased owing presumably to the greater resistance to flow offered by collapse of the veins as they entered the thorax. Changed volume flow was not responsible (137). Raising intrathoracic pressure to the atmospheric level and above, by breathing against positive pressures, increased auricular pressure more rapidly than femoral venous pressure, and reduced the gradient until both pressures became almost equal at +10 cm. water when the veins were distended and offered least resistance to flow. Similar experiments in the normal supine human being (136) suggested that in man the veins may not collapse so easily perhaps because the right auricular pressure of man is slightly above atmospheric pressure (138, 139). Yet, in man the average resting gradient between peripheral venous and auricular pressures still ranged from 12 to 76 mm. water with an average of 39 mm. (138, 139). Acute cardiac failure in animals (138, 140) and chronic congestive failure in man (139) reduced this gradient to a few millimeters of water. Hence, in man as in animals, the first small elevations of auricular pressure elevate venous pressure but not by exactly corresponding amounts. The gradient itself varies widely in normal subjects and is also too great to be caused by differences in blood flow alone.

This variability of gradient is, no doubt, one of several reasons for the failure of resting peripheral venous pressures to reveal the very early stages of cardiac failure in patients. Fortunately, these factors become less important as auricular

pressure rises further and as the veins become tense and fully distended. Under these conditions antecubital venous pressures can be used to estimate changes in auricular pressure with sufficient accuracy to be clinically helpful. Even so, the danger of greater venous resistance in the axillary region requires careful placing of the arm to avoid erroneously high readings (141, 142).

Starling's heart-lung preparation (2, 3, 143, 144) eliminated the complications of venous resistance, intrathoracic pressure, vascular volume, changing heart rate, neurogenic factors and hormonal influences that make studies in the intact animal so difficult. This simplified and controllable circulation revealed clearly the rapidity and accuracy with which the isolated heart can adapt, within physiological limits, to a changing rate of inflow from the veins or to increased resistance of outflow, by correspondingly changing its energy of contraction. Although the observed relation between auricular pressure and the corresponding stroke volumes was generally close, Starling chose to word the 'Law of the Heart' in more general terms, and related the energy of contraction to the length of the muscle fibers without including, in the 'Law' at least, any specific reference to venous and filling pressures. Nevertheless, by the use of more accurate methods of measuring pressures continuously throughout the cardiac cycle, the importance of diastolic filling pressures was demonstrated under diverse conditions by Wiggers and his associates (145–147). Similar conclusions have resulted from myographic studies of the exposed heart (148).

In the heart-lung preparation the behavior of auricular pressures, as inflow of blood is increased, also provides an extremely useful method of measuring spontaneous or induced impairment of cardiac function, its improvement by drugs and its relation to induced changes of heart rate (149, 150a). In such preparations stepwise increases of the volume of blood supplied to the heart in unit time (produced by increasing, in steps of 50 mm., the height above the heart of the venous reservoir and its blood level) raise auricular pressure by only a few mm. water with each increment, so long as the volumes presented to the heart fall within the heart's 'competence' (Leistungsfähigkeit), i.e. the range of work load through which it can increase its output to match the volume supplied. Finally, however, as the reservoir is raised still further, the volume of blood that can be supplied from the reservoir becomes so great that the heart's 'limit of competence' (Suffizienzgrenze, 150b) is approached or exceeded. Then the rise of auricular pressure, per 50 mm. increase of reservoir height, becomes much larger, e.g. 30 to 50 mm. water, or even more, and the gradient of pressure between the venous reservoir and the auricle does not increase further or even diminishes. The pressure-flow characteristics of the tubing between the reservoir and the auricle make it possible to imitate in suitable preparations the relation between peripheral venous and auricular pressures reported during cardiac failure in animals and man (138–140) where the gradient also becomes less as cardiac competence declines. For the dog's heart, the approximate degree of reduction of competence has been expressed in terms of the 'competence index' (151) which is the ratio

$$\frac{\text{increase in inflow level} - \text{increase in right atrial pressure}}{\text{increase in inflow level}}.$$

This index ranges from 1.0 for a fully competent heart down to 0.0 for a fully incompetent heart. Collectively, these and many other studies on the heart-lung preparation have not only demonstrated the relation of filling pressure to cardiac output but have also made clear the difficulty of distinguishing the pressure and volume dynamics of a slightly or moderately damaged heart from those of a normal heart if the imposed burden (volume of inflow or resistance to output) is small. Striking rises of atrial pressure appear, however, when the burden equals or exceeds the heart's 'limit of competence.'

The technique of venous catheterization has made it possible to study the relation between auricular pressure and cardiac output in human beings with normal and abnormal cardiac function. Most investigators (152–159) have found so far that the relation between auricular pressure and cardiac output in man generally conforms, in a wide variety of conditions, to that previously described for the heart-lung preparation, emphasizing the importance of filling pressures not only when the heart is competent (159) but also when it is grossly incompetent (156). Others have found under some conditions that cardiac output could be altered without concomitant changes of auricular pressure (160, 161), particularly when cardiac function is still relatively normal. Even when venous and auricular pressures are elevated, cardiac output may be low, normal or abnormally high (154, 155, 162). Reducing auricular pressure by venesection lowers cardiac output in normal subjects but increases output in so-called 'low output heart failure' (156). The latter appears at first sight to be a paradox but is really quite similar to the behavior of the pathologically dilated heart in the heart-lung preparation.

Conditions in the intact organism are so complex as to preclude any simple general formula nor is there enough information at present to describe precisely the limits within which a given simple formulation will always apply. First, in the presence of normal cardiac function, interpretations have had to be based upon relatively small changes of auricular pressure with methods which still have limitations. In all but a few studies auricular pressure has been measured against a constant atmospheric pressure, whereas the walls of the large veins and cardiac chambers are exposed to an intrathoracic pressure which is not only subatmospheric but also changes phasically with respiration. Burton-Opitz (29) observed that opening the chest raised general venous pressure, and later Henderson and Barringer (163) and Wiggers (164) emphasized the important fact that effective filling pressure of the heart is not determinable simply by manometers equilibrated with atmospheric pressure but that it must be calculated as the algebraic difference between pressure within and around the atria. Moreover, it has been shown recently that intrathoracic pressure is not the same in all regions of the thorax and the exact value to be used in calculating effective filling pressure is still under study (165, 166).

Second, it may be inquired whether lengthening or shortening of the cardiac muscle fibers must necessarily in all instances, and in the intact animal, be accompanied by an easily measurable change in central venous or atrial pressure. The freshly prepared mammalian heart-lung preparation, despite its remarkable performance, has already suffered somewhat from preliminary anesthesia, surgical exposure and manipulation as well as perfusion with blood which is not entirely normal.

The life span of a mammalian heart-lung preparation is limited and, even under the best conditions, spontaneous reduction of its competence index from initial levels between 0.71 and 0.95 to between 0.13 and 0.29 can occur within 2 to 5 hours (151). This is accompanied by the necessity of using larger and larger increments of auricular pressure to accomplish a given work level. The more rugged turtle heart, contracting isometrically, reached its maximum systolic tension while diastolic pressure changed from zero to less than 2 mm. Hg (167). Higher diastolic pressures did not produce further increase of systolic tension and eventually, as diastolic pressure began to rise sharply, systolic tension fell off considerably. More relevant to the mammalian circulation is the observation by Opdyke *et al.* (168) that the large capacity of the right atrio-caval system permits moderate and small changes in the volume of venous return without measurable differences in right atrial pressure, although pressures within the less distensible left atrium are affected. It may well be in the intact human being that large, sustained changes in venous return are more apt to affect atrial pressure while smaller transitory changes may not.

Third, in the intact organism, the effects of innervation (169) and of epinephrine (150a, 170–171) as well as other possible humoral agents may grossly shift the curve relating cardiac output to diastolic volume or filling pressure. This shift may even be quantitatively different for the right and left ventricles (172). In moderate and severe exercise the cardiac volume of normal subjects usually increased but exceptions were noted; Starling's law can apparently be modified so that a given stroke volume may be obtained with less cardiac dilatation than during rest (173, 174). For these reasons the relation between moderate changes in venous return, auricular pressure and cardiac output of the normal human heart will quite properly continue to be a matter of experiment and debate for a long time to come. Richards (175) has summarized the general problem:

> Starling's law, in fact, applies to the human circulation about as well as one could expect of a physiologic principle developed from the performance of a heart-lung preparation; the more the human heart is exposed to and reacts to nervous, vasomotor and metabolic influences, the less it behaves in accordance with this law; the more the heart fails to respond to such influences and becomes simply a pressure-volume muscle preparation, as in congestive failure, the more closely it follows the law.

Even though the uncertainties listed above do not interfere quite so much with interpretations of the somewhat larger changes of venous and auricular pressures observed when cardiac competence is decreased, the relation between high venous pressures and cardiac function has also been the subject of debate. This arose largely because excessive emphasis was placed on resting venous pressures with neglect of the important demonstration in the heart-lung preparation that a moderately damaged heart accomplishes its task with normal, or almost normal, filling pressures and fiber length until the load of venous return is made great enough to reveal the lowered 'limit of competence' which was present, but latent, before the period of stress (143, 144, 150). Starling (176) regarded the elevated venous pressure in patients with heart failure as a direct effect of decreased cardiac function. This concept with its tacit assumption that 'back pressure' occurs in the intact animal, as it does more clearly in the heart-lung preparation, was challenged because of the constancy of resting venous pressures in animals and patients with known myocardial injury.

Starr *et al.* (177) found in dogs that acute injury produced by burning the surface of the right ventricle, or by ligation of superficial coronary vessels, failed to elevate femoral venous pressure, but these studies were limited to the resting state and to peripheral, rather than central, venous pressures. Warren and Stead (178) withheld mercurial diuretics from two patients recovering from severe congestive failure and then administered water and sodium chloride. Observing that blood volume, measured by dye, and body weight increased before peripheral venous pressure rose, they concluded that the earliest manifestation of cardiac insufficiency must be renal retention of fluid and an increase of blood volume. According to this view (177, 178), plethora or hypervolemia is responsible for the elevated venous pressure which becomes then a remote and indirect, rather than an immediate and direct, result of right ventricular failure. The results of Warren and Stead (178) may have been due chiefly to the sudden withdrawal of a powerful diuretic agent or their two patients may have been exceptional examples. Reichsman and Grant (179) found that withholding digitalis from a larger number of patients partially recovered from congestive failure was followed first by elevation of venous pressure and then by gain in body weight and finally by the appearance of edema. Threefoot *et al.* (180) and Gibbons (181) observed in another series that body weight and venous pressure generally rose and fell together during increasing decompensation and recovery, though transitory exceptions were noted in which the state of the tissues, and possibly tissue pressure, seemed to explain prior changes of one or the other. Slight changes of venous pressure, even within the so-called normal range, were associated with significant changes of body weight. In the cardiac patient it is difficult to determine the exact relation between resting peripheral venous pressure and plasma volume. Though one might be closely related to the other, it is also possible that both are collateral changes and, in varying degree, due to a common and more fundamental abnormality. Both tend to increase as cardiac failure involves the right side of the heart, and both change much less conspicuously in left heart failure, though in the latter pulmonary venous pressure is high. Even in advanced congestive heart failure they do not increase and decrease in strict proportion (182, 183); a slight negative correlation has been found in one series (183). In pregnancy blood volume is increased for some months (184) but resting antecubital venous pressure remains statistically constant (185) and within normal limits even in the presence of mild cardiac failure (186).

The essential questions can be listed in sequence. *a*) What is the relation in the intact animal between cardiac damage and venous or auricular pressure at rest? *b*) If damage of the right ventricle does not elevate central venous pressure during rest, will muscular activity, by imposing a heavier load of venous return on the heart, raise central venous pressure to a greater degree than similar exercise does in normal animals? *c*) If muscular activity characteristically elevates central venous pressure, slightly or markedly, in the presence of reduced cardiac competence and normal blood volume, will a simple increase in blood volume per se duplicate this effect when the heart is normal? *d*) If cardiac competence is reduced, what is the effect of increased blood volume on venous and auricular pressures?

Landis, Brown, Fauteux and Wise (35) found in normal, anesthetized dogs that muscular contractions, produced by electrical stimulation of four extremities, increased respiration and reduced central venous pressure. In agreement with others

(177, 187) they found that coronary ligations and auricular fibrillation did not elevate resting venous pressure. However, in such animals when venous return was increased by muscular contractions in four extremities, central venous pressure rose absolutely or relatively and remained above the control level as long as muscular activity was continued. This relation between reduced cardiac competence, exercise and venous pressure has been shown far more clearly by Barger *et al.* (188) in dogs surviving the surgical production of pulmonary stenosis and tricuspid regurgitation. Prior to operation, control observations in these dogs showed that auricular pressure did not rise significantly during exhausting exercise on the treadmill. Post-operatively, resting auricular pressures were moderately elevated but exercise to exhaustion increased auricular pressures to the remarkable levels of 350 to 450 mm. water. At this stage exercise tolerance was greatly limited. The answers to questions *a*) and *b*) appear to be in accord with results obtained in the heart-lung preparation. Moderate decreases of cardiac competence do not elevate resting auricular pressure in the intact animal. On the other hand, when the stress of increased venous return approaches the 'limit of competence' of the heart, auricular pressure rises. In acute experiments 'limit of competence' must be decreased almost to lethal levels before resting auricular pressure rises. This was observed after coronary ligations in only one dog (35) and then for only a few minutes before fatal ventricular fibrillation occurred. When diffuse coronary occlusion was produced (189) by starch granules, resting venous pressure rose more markedly and finally arterial pressure declined. The elevations of auricular pressure produced in all of these acute experiments (35, 189) were moderate, as might be expected with a normal blood volume, operative trauma, blood loss, and a distensible venous system.

The effect of plethora per se has also been explored in normal dogs and in dogs with acutely and moderately reduced cardiac competence. In the former, Meek and Eyster (190) showed that the diastolic volume of the heart increased during infusions while venous pressures rose to 150 mm. water, but that return to normal was rapid. Very rapid and large infusions produced signs of congestive failure but the effect on resting auricular pressure was temporary (191). Increasing blood volume 50 per cent by rapid transfusion of whole blood elevated the resting venous pressure of dogs temporarily by 7, 14 and 23 cm. water but within 7 to 20 minutes after the transfusion ended venous pressure was again normal (35). Warren, Merrill and Stead gave 3 normal dogs intravenous infusions of physiological saline solution equivalent to their body weight, i.e. at least 10 times the estimated blood volume; one hour after the infusion ended, venous pressure was normal in 2 dogs and 8 cm. water higher than the control level in 1 (126). During and after such acute elevations of venous pressures by massive infusions or transfusions, muscular activity did not produce any further increase of central venous pressure such as was seen during muscular activity with normal blood volume when cardiac competence had previously been reduced (35). To maintain resting central venous pressure at levels of 50 to 100 mm. water above normal required tremendous and wholly unphysiologic rates of infusion or transfusion. Even during this period of sustained venous hypertension muscular activity did not increase venous pressure (35). It can be concluded that simple plethora in the presence of normal cardiac competence has not so far duplicated the effects which reduced cardiac competence produces even when blood volume is normal (35).

In the presence of reduced cardiac competence plethora accentuates the rise of auricular pressure (172, 189), reduces the amount of trauma required to produce a given rise of auricular pressure (189) and may produce dissociations between right and left ventricular function (172). It is obvious that more experiments of this nature are needed to bridge the remaining gaps between the acute heart failure which can be produced in the laboratory and the more chronic end results of heart disease seen in resting or active patients. At the present time the available results agree, within reasonable limits, with those of the heart-lung preparation and the effects of exercise on venous pressure offer explanations for certain puzzling clinical observations which are mentioned later.

VENOMOTOR TONE

Of all the factors which contribute to the maintenance of venous pressure least is known definitely concerning the quantitative significance of venomotor tone. Despite this the term has sometimes been used with the unproved connotations, tacit or expressed, *1*) that the veins by their general contraction can per se elevate venous pressure and *2*) that the veins often react as an independent unit of the vascular system. This is doubtless due in part to a natural tendency to assume that venous tone and venous pressure are related to each other in the same general fashion that arteriolar tone and arterial pressure are related. Careful comparison of these two systems reveals interesting differences.

Arterial pressure is measured midway between the heart and the contractile arterioles; the latter by their position and resistance (with that of the arteriovenous communications and capillaries) control outflow from the arterial system and hence affect arterial pressure directly. Venous pressure, on the other hand, is measured in veins which are themselves contractile and midway between the contractile venules and the heart. Again the latter is in a position to control outflow from the venous system which leads to the interesting analogy that the heart must be an important part of the 'peripheral resistance' of the venous system and, other things remaining constant, changes in this 'cardiac resistance' should affect venous pressure in the same direction as changes in arteriolar resistance affect arterial pressure. This leaves to venomotor tone a mechanism of function very different from that of arteriolar tone.

That veins are contractile is well established by abundant evidence; the tension of their mural smooth muscle can be changed by mechanical, thermal, chemical and either direct or reflex nervous impulses (192; for reviews see 10–12). The most important effect of generalized venous dilatation or constriction will be respectively to increase or decrease vascular volume. The effects of increased vascular volume have been described previously in detail; dilatation of venules and veins can reduce peripheral and central venous pressure conspicuously not by merely reducing the resistance to venous flow, which is situated in large part in the heart, but by expanding the volume of the peripheral vascular bed and by reducing the effectiveness of *vis a tergo*. Thus, while reduction of venomotor tone reduces venous pressure, its mechanism of so doing is quite different from that by which reduction of arteriolar tone reduces arterial pressure.

Conversely, the most important effect of widespread venous constriction must

be the reduction of venous vascular volume which can have two consequences, *1*) to increase the volume of venous return temporarily by a corresponding volume of blood or *2*) to raise central venous pressure, if the heart cannot accept the added venous return because it is unable, through reduced competence, to elevate its output correspondingly. Thus, the immediate effects of a simple increase of venomotor tone would be comparable to those of an intravenous injection or infusion which affects venous pressure less or more depending upon whether cardiac competence is normal or is less than normal.

The earlier observations of Heymans, Bouckaert and Dautrebande on the carotid sinus and moderator mechanism (193) have been extended recently by Charlier (194, 195) with particular reference to auricular pressure and cardiac output, using the Fick method of determining the latter. Clamping both carotid arteries, to decrease pressure in the carotid sinus, increased auricular pressure by 2.5 to 15 mm. water in 18 of 21 experiments with increases of 20, 25 and 90 mm. water in the remaining three experiments.[3] Cardiac output increased by 9 to 160 per cent with an average of 48 per cent and arterial pressure rose by 13 to 69 per cent. Average total peripheral resistance throughout the body sometimes increased and sometimes decreased. Charlier ascribed the rise in cardiac output to reflex venoconstriction, to shifts of arteriolar resistance in various organs with opening of arteriovenous communications (193) and to increased venous return to the heart. Auricular pressure rose very slightly in most instances despite considerable increases of cardiac output, elevation of arterial pressure, and greater venous oxygen saturation. In the resting animal, according to Charlier, the carotid sinus and the aortic nerves tend to reduce the cardiac output. By this concept the regulation of arterial blood pressure and venous pressure are closely allied through a cycle which involves *1*) the well known sensitivity of the moderator nerves to alterations of arterial pressure (193), *2*) the reflex changes of the tone of arterioles and arteriovenous communications (193), *3*) the tone of the venous vessels which through changes of their volume can either make available, or sequester, significant volumes of blood, and *4*) the competence of the heart which functions normally at low venous pressures.

Holt *et al.* (195a) stimulated the carotid sinus nerve and concluded that the fall in arterial pressure thus produced was caused to a large extent by a decrease in cardiac output, and to a small extent by decreased peripheral resistance. They stressed the important effects which changing tone of the smooth muscles of the veins (post arteriolar blood vessels), with consequent changes of venous vascular volume, must have on venous return to the heart. More studies on this important point are needed, because others (193, 193b) have not observed consistent changes of cardiac output when pressure in the carotid sinus was modified.

The second difficulty encountered in the uncritical use of the term venomotor tone is the inability, in many instances, of distinguishing clearly between the re-

[3]These quoted values are in accord with Charlier's text (195) and also with table 1 because the headings for right auricular pressure and right ventricular pressure in the left half of table 1 should be reversed (personal communication).

actions of arterioles, venules and veins. In exceptional circumstances such separation can be accomplished, e.g. during the action of sodium nitrite in which abnormal pooling of blood in congested veins was found plethysmographically despite compensatory arteriolar vasoconstriction identified by decreased blood flow (85–87). Under the influence of nitrite the venules did not respond as vigorously to epinephrine as the arterioles (86). Strictly independent reactions of the veins are relatively few. Veins participate with the arterioles in the reactions to CO_2 and bicarbonate (196) and to inhalation of CO_2 (192, 197, 198). Fleisch (199) observed increased venous return to the heart when the pressure in the carotid sinus was reduced, and decreased venous return when pressure in the carotid sinus was increased. Removal of blood and infusions respectively constricted and dilated the mesenteric and colic veins (200). Gollwitzer-Meier in her excellent review concludes that the close relation between changes of arteriolar and venous tone bespeaks a simultaneous spread of vasomotor impulses to arterioles and veins. This is seen best in the simultaneously rhythmic swings of arterial and venous pressure when respiratory variations have been suppressed. In asphyxia, hypercapnia and hypoxia, arterial pressure rises and arteriolar tone increases but the veins constrict also. In reflex vasodilatations the converse usually holds, and clear separation of venomotor responses is most difficult. While the term venomotor tone suggests an autonomy greater than that which really exists, its continued use may be justified if it is understood that venomotor tone changes venous vascular volume more than it modifies resistance to flow, and that a rise of venomotor tone per se cannot produce a rise of venous pressure except briefly. In this sense decrease of venomotor tone permits the volume of the vascular system to expand and accommodate considerable volumes of fluid or blood without persisting change in venous pressure providing cardiac competence is normal. This has already been discussed in the section above in terms of infusions and transfusions.

Of equal importance is the reduction of venous volume that can be produced by increased venomotor tone. The volume of blood thus shifted from the veins may increase cardiac output significantly (195) and should, in emergencies, help compensate for sudden reductions of blood volume or effective blood volume. The maximum possible decrease of vascular volume was studied by Riml (201) who clamped the pulmonary artery of rabbits and found that about 50 per cent of the whole blood volume would flow from the venous system at pressures of 0.5 to 1 cm. water. This figure represents an extreme value because it includes the effects of agonal vasoconstriction, compression of veins by convulsive contractions of skeletal muscle (132) and a reduction of central venous pressure below normal cardiac filling pressure. When venous pressure was held at its normal resting level during the final asphyxia of dogs the volumes expressed at that pressure from the vascular system ranged from 21.0 to 22.4 cc/kg. (35). Assuming total blood volume to be 8 per cent of body weight this would correspond to a decrease of vascular volume by 26 to 28 per cent at normal cardiac filling pressure. The corresponding figure for a man weighing 75 kg. would be approximately 1600 cc. Changes in blood volume before death probably modify this figure considerably because in 2 dogs after several periods of high venous

pressure, due to combined auricular fibrillation and muscular activity only 9 and 11 cc/kg. were expressed, and in a third dog after infusion of 3600 cc. of Ringer's solution and similar exercise, 40 cc/kg. were expressed (35).

By a very rough estimate, based on these few observations, it appears that approximately half the blood volume occupies what might be termed the vascular 'dead space' which remains filled with blood at zero venous pressure even during agonal vasoconstriction and convulsive muscular contractions. Approximately a quarter of the blood volume is required in addition, even during agonal vasoconstriction, to fill and distend the vascular system sufficiently to maintain a normal venous pressure and a normal cardiac filling pressure. The remaining quarter of the blood volume represents the amount that can be made available through changes in venomotor tone to compensate for blood loss or for pooling of blood in the veins whenever they are distended locally by venous pressures above normal. In this connection it may be recalled that in dogs the heart retained its usual diastolic size and output until the blood lost by hemorrhage equalled about 21 cc/kg. body weight (202) and that in man acute loss of blood amounting to 1 liter, or about 15 cc/kg. body weight (203) is the point at which mild or severe symptoms begin to appear intermittently even in the recumbent subject.

Finally, the combined interactions of arteriolar tone, venomotor tone, venous pressure and blood volume are all essential parts of the profound physiological readjustments which, in an intact animal, permit the maintenance during cardiac failure of a high venous pressure and a normal arterial pressure, despite the tendency of a high venous pressure to make the animal bleed into its own distensible venous system (176). The fundamental importance of these active physiological responses makes it necessary to recognize the limitations of carrying over to the intact living animal the pressure relations found in simplified circulation schemata (204, 205) and in dead animals (206). During life arterial pressure and venous pressure are both kept relatively constant by the moderator and probably other reflexes, but the effectiveness of these reflexes depends upon the ability of the normal heart to change its output greatly with minor changes of venous and filling pressures. Cardiac resistance to inflow of venous blood (which, to paraphrase from Meyer's (205) electrical analogy, is effective auricular or filling pressure ÷ cardiac output per unit time) is normally low and interposes only a slight barrier between the venous and arterial sides of the circulation. Each side can respond in sensitive manner to changes in the pressures and volumes of the other so long as the heart retains its remarkable ability to adjust rapidly to varying venous return with little or no change in filling pressure.

As cardiac competence is reduced, however, rising cardiac resistance to flow probably places an increasingly important barrier between the venous and arterial sides of the circulation. Nevertheless, arterial pressure is still kept relatively constant until very severe cardiac failure develops. In this readjustment, the constancy of venous pressure, and later the constancy of blood volume, are apparently sacrificed to the more basic requirement of a sustained arterial pressure and cardiac output. To disentangle the mechanism, timing, magnitude and relative importance of the complex reflex and hormonal adjustments which determine ar-

teriolar tone, venomotor tone, venous pressure and eventually blood volume in acute and chronic cardiac failure, during exercise and rest, is a formidable task for which many more data are required.

LIMITATIONS OF VENOUS PRESSURE MEASUREMENTS

Circulatory function is so important in all branches of medicine and surgery that as soon as a simple direct method gave promise of providing immediate and helpful information at the bedside it was eagerly and generally used. Resting venous pressure has been measured in a great variety of clinical conditions (9, 12–14, 207), complete tabulation of which is beyond the scope of this review. From these empirical observations, with the help of analytic studies, it is possible, however, to begin to outline the factors which limit, and those which may increase, the usefulness of determinations of venous pressure in the study of specific functions.

Variability of Resting Venous Pressure and its Relation to Low Venous Pressures. Even in recumbent subjects, in a resting state physically and mentally, the absolute values of peripheral venous pressures vary widely from person to person. This variation has been ascribed chiefly to the collapsibility of proximal veins (105) and to the difficulty of establishing a suitable zero point for reference (6, 7, 142, 208–210). Of these zero points the one recommended by Lyons, Kennedy and Burwell (142) provided good agreement with the position of the auricle when checked by x-ray or auricular catheter (139). Nevertheless, by this method as well as by that of Winsor and Burch (210) normal venous pressure in the antecubital veins ranges from 50 to 140 or 150 mm. water, a spread of 300 per cent. The lowest values are very close to the range of 20 to 50 mm. water which is the residual pressure (70, 62) and which persists as the arm is raised above the clavicle.

It is important to note that resting auricular pressure also varies from subject to subject, e.g. 10 to 75 mm. water, and that the gradient between antecubital venous and auricular pressures ranges from 12 to 76 mm. water. A six-fold spread of gradient cannot be explained by differences in resting blood flow but might well be due to the varying resistance of more or less collapsed veins. While differences, from subject to subject, of mean intrathoracic pressure could hardly be this great during quiet respiration, it is possible that mild excitement might affect breathing and so produce abnormal intrathoracic pressures in some subjects. In any case the variability of auricular pressure itself again invites speculation *1*) concerning the relative importance of fiber length or filling pressure in the entirely normal heart and *2*) concerning cardiac 'tonus' (211–213). Before any conclusions on these highly debatable issues can be reached, it will be necessary to have many more simultaneous and highly accurate records not only of auricular pressure but also intrathoracic pressure near the heart similar to those of Lausen *et al.* (159). At present the variations of normal, resting, auricular pressure seem to be as inexplicable as the normal variations of normal, resting, arterial pressure.

With such spread of peripheral venous and auricular pressures, when determined against atmospheric pressure, it is not surprising that measurements of peripheral venous pressures offer little assistance diagnostically in the early or late stages

of those conditions, e.g. hemorrhage and shock, which tend eventually to produce visible collapse of veins and low auricular pressures. Experimental results also provide little reason to believe that moderate reductions of blood volume can be detected with certainty by reductions of peripheral venous pressure. The moderator system, by the cycle described in the previous section of this review, probably compensates for blood loss by reducing venous vascular volume so that both arterial and venous pressures are kept as close as possible to normal until final collapse. It has been suggested that frequent measurements to detect small relative changes might be more helpful than isolated absolute values, but this seems doubtful when the removal of approximately 1 liter of blood (circa 15 cc/kg. body weight) from normal subjects produced no change or only a small decrease of peripheral venous pressure (203). In dogs Meek and Eyster (202) found that hemorrhage amounting to 20 cc/kg. body weight produced only transient effects on arterial and venous pressures, and diastolic heart size. Moreover, when hemorrhage was severe enough to produce shock, Wiggers (214) found that central venous pressures (i.e. atrial pressures, corrected for simultaneous intrathoracic pressures) were affected oppositely by 2 factors, decreased venous return and progressive myocardial failure.

Comparative Significance of Venous Pressures Determined During Rest and During Stress. Determinations of venous pressure have found their greatest clinical usefulness in the high values found locally when a large vein is partially or completely obstructed, and found generally throughout the body when advanced cardiac failure is present. In order to reduce normal variability to as narrow a range as possible, peripheral venous pressures have generally been measured during absolute rest. By reducing venous blood flow to a minimum this produces constancy but diminishes the likelihood of detecting slightly or moderately increased resistance to venous blood flow, and correspondingly limits the information to be obtained.

Marked local elevations of venous pressure by thrombophlebitis, or by compression of veins by neoplasms are familiar and established findings, though collateral veins permit complete obstruction of smaller veins, or partial obstruction of larger veins, to exist without elevating venous pressure below the lesion to clearly abnormal levels. In such instances Veal and Hussey (68) found that muscular contractions, by increasing blood flow, elevated a presumably normal resting venous pressure to very high levels which were clearly beyond normal range, e.g. from 130 mm. water to 900 mm. water. This emphasizes the important fact that venous pressure is a function not only of the resistance to flow which is situated beyond the point of measurement but also of the quantity of blood flowing per unit time as indicated by rearranging Poiseuille's equation:

$$Q = \frac{(P_1 - P_2)r^4 t}{8l\eta} \quad \text{to} \quad P_1 - P_2 = \frac{Q}{t}\frac{8l\eta}{r^4}$$

With constant radius r, effective viscosity η, and length l, the difference of pressure $(P_1 - P_2)$ on the 2 sides of the obstruction, and hence the venous pressure measured distal (anatomically) to the obstruction, will increase as the volume flow increases. Normally, the total cross-sectional area of the veins is so great that flow can be increased several fold (as mentioned under *vis a tergo*, in the second section of this review) without raising venous pressure by more than a few centimeters of

water at most. If resistance to flow is increased, however, the effects of changing blood flow are magnified and it is possible to detect an obstruction which was not great enough to be identified with certainty at lower, resting venous blood flows. This has been considered in some detail because of the interesting resemblance between the simpler situation of a partially obstructed vein and the far more complicated situation of a heart with reduced competence. In both, venous pressure may be within the rather wide limits of the 'normal range' (50 to 150 mm. water) but in both, increasing the rate of venous return can elevate venous pressure beyond the normal range and so reveal a resistance to flow which could not otherwise be detected.

That this limitation of resting venous pressure has been recognized sporadically in clinical studies, as well as in the laboratory, is shown by the published descriptions of test procedures designed to increase temporarily the volume of blood returned to the heart per unit time, and thus add greater precision to the use of venous pressure in assessing the earlier stages of reduced cardiac competence. This has been done by measuring venous pressure during muscular activity (215–219), pressure on the upper abdomen (220), administration of intravenous infusions (221–223) and passive elevation of the lower extremities (221, 224). In these observations some patients with 'compensated heart disease' had normal venous pressures at rest when venous return was small but exhibited abnormally high venous pressures during, and for some time after, periods of increased venous return. In a comparative study of these procedures Hultgren (225) found recently that they did not significantly affect peripheral venous or right auricular pressures of normal subjects. In 'left heart failure' the effects of volume stress on venous pressure were peculiarly biphasic, slight or absent. In 'right heart failure' increasing venous return produced elevations of 20 to 140 cm. water. As might be expected the effects were smallest and most transient with leg raising, somewhat larger and more persistent during moderate exercise in bed and during abdominal compression. Of particular significance were the elevations of 20 to 120 mm. water seen in patients with resting venous pressures of 150 mm. water or less. Similar results were obtained when auricular pressures were measured; the correspondence between them and peripheral venous pressures was surprisingly good. Hence in the failing heart-lung preparation and in dogs and patients with reduced cardiac competence, an increased venous return can produce transitory elevations of peripheral venous, central venous and auricular pressures, even though these pressures were previously, during rest, within the so-called normal range.

The effects of exercise on venous pressure in normal subjects suggest that the difference between them and patients with overt cardiac failure may be quantitative rather than qualitative. Exercise elevates peripheral venous pressure in proportion to work intensity (226, 227). At exhaustion, antecubital venous pressures were 80 to 110 mm. water above the control resting level (227) but whether similar elevation of auricular pressure occurs in normal subjects under these extreme conditions is still unknown. Enlargement of the liver has been found in poorly conditioned athletes after they have run long distances (228). Exercise is also often accompanied in normal subjects by decreased renal blood flow (229–231) and, as exhaustion approaches, by cutaneous vasoconstriction (227).

Venous Pressure and Edema. Other factors remaining constant, acute elevations

of venous pressure increase the filtration of fluid from the capillary blood into the tissue spaces approximately in proportion to the rise of venous pressure. An equally close correspondence between high resting venous pressure and the amount of edema is not found (207) and cannot be expected because the amount of fluid in the tissue spaces at any given time depends on other factors as well. Venous pressures measured in cardiac patients at absolute rest may be very much lower than those existing during prior activity. Differences of tissue pressure and the flow of lymph may also produce discrepancies. Ligation of the inferior vena cava produces edema in the lower extremities but with the passage of time this edema tends to lessen as venous pressure diminishes, and may even disappear before venous pressure has returned to normal (232a, b). This suggests the development or widening of collateral venous channels and also the possibility that, with varying rapidity in different subjects, the removal of excess fluid via the lymphatics becomes more efficient.

While the relation between venous pressure and filtration of fluid through the capillary wall is unquestioned, an acute, general rise of venous pressure throughout the body, such as that which probably occurs intermittently in the active cardiac patient, involves much more than simple filtration of fluid. Concurrent pooling of blood in the congested veins simultaneously tends to reduce effective blood volume. The amount of fluid filtered in any brief period of elevated venous pressure is an important reduction of plasma volume but an insignificant increase of overall tissue fluid volume. In contrast to small, localized collections of edema fluid, the mere volume of the massive edemas of heart disease, nephritis and nephrosis necessarily requires, in addition to filtration, the progressive retention in the body of successive increments of sodium chloride and water over periods of days or weeks. Peters (233) has pointed out in detail the serious difficulties that are encountered in attempting to explain cardiac edema on the basis of 'forward failure' of the heart (178) which assumes the sequence, *a*) renal retention of salt and water, *b*) expansion of plasma volume, *c*) increased venous and capillary pressure and transudation. The evidence as a whole favors the sequence *a*) increased venous pressure and capillary pressure, *b*) transudation, *c*) hemoconcentration and *d*) retention of salt and water. It is still possible that the older explanation of cardiac edema by 'pre-renal deviation' of sodium and water, owing to high venous pressure and filtration into the tissue spaces, with secondary (and for plasma volume, compensatory) retention of sodium and water by the kidney is not so much wrong as it is oversimplified and too general because it does not imply or indicate the intermediate steps by which the kidney is stimulated to retain rather than excrete. In recent reviews of the role of the kidney in congestive heart failure it was not possible to establish clearly the mechanism by which renal retention of sodium and water is produced even in this one disease (234, 235) though elevated renal venous pressure and reduced glomerular filtration both occur. Oliguria during and after quiet standing on the tilt table (236, 237) as well as the decreased renal excretion of both sodium and water during exercise of normal subjects (238) or following congestion of three extremities (239) suggest that this response is not uniquely limited to patients with severe cardiac failure, but may be produced in normal subjects by suitable circulatory stresses.

CONCLUSION

Consideration of the multiple, interlocking factors which affect, and are affected by, venous pressure reveals how limited is our quantitative information concerning this commonplace, almost trite, measurement. Appreciation of the role that venous pressure plays in the circulatory adjustments of the intact animal has probably been made more difficult than necessary by placing undue emphasis on the convenient constancy of measurements taken only during rest. Certain differences between cardiac failure in the heart-lung preparation and cardiac failure as seen in intact animals or patients may seem impressive if, in the latter, measurements of venous pressure are limited to the resting state. However, when the volume of venous return per unit time is increased, e.g. by exercise, and when central venous pressures, or better still effective filling pressures, are measured, similarities become more prominent. Such differences as persist, despite otherwise similar experimental conditions, will bring into sharper focus the possible influences of innervation and of hormones on the relation between venous pressure and cardiac function of the intact animal.

Brief, generalized elevations of venous pressure not only decrease plasma volume and produce hemoconcentration by filtration of fluid but tend also to reduce effective blood volume by greater filling of the normally capacious venous system. More sensitive methods of measuring changes in effective blood volume would be most helpful because a given increase of the volume of the venous system can, unless promptly compensated, produce a proportionately greater decrease of the smaller volume of blood available to the arterial portion of the circulation. Closely allied to these changes is the difficult problem of the relation between venomotor tone and the volume-pressure curve of the entire venous system during various circulatory stresses.

The degree to which the moderator system of reflexes can ensure constancy of venous, as well as arterial, blood pressure needs further study, particularly because of the possibility that reduced cardiac competence, by increasing cardiac resistance to the flow of venous blood, may interfere with an otherwise sensitive mechanism. With these factors in mind, more experiments on intact animals with normal and reduced cardiac competence, during rest and graded exercise, are needed to clarify the relation between repeated, temporary elevations of venous pressure during activity or change of posture and the fully developed syndrome of advanced congestive heart failure as it appears in man with its confusing tetrad of high venous pressure at rest, edema, modified renal function and plethora.

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GASTROINTESTINAL HORMONES

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IT IS NOW 20 years since the last review with this title appeared in this Journal (216). During this period important advances have occurred; new hormones have been discovered, others have been purified and the mechanisms of their action have been further elucidated. Work reviewed by Ivy (216) will be treated here only in those instances where new knowledge has led to reinterpretations of the older evidence. A number of review articles of varying degrees of comprehensiveness have appeared during the last 20 years (22, 164, 165, 209, 218, 220, 221). For the purposes of this review the gastrointestinal hormones will be limited to those hormones which are produced by the gastric and intestinal mucous membranes (thus insulin is excluded). Certain substances whose hormone status is uncertain, namely, urogastrone and anthelone, will be considered because of the intimacy with which their development has been related to studies on gastrointestinal hormones.

Terminology. Ivy (216) favored the use of the nomenclature proposed by Sharpey-Schafer (412) in which the general term 'autacoid' was subdivided into 'hormones,' or excitatory autacoids, and 'chalones,' or inhibitory autacoids. Since this system of nomenclature has not gained currency, in this review the term 'hormone' will be used in its usual general sense of a physiologic organic compound produced by certain cells and carried by the blood to distant cells, the activities of which it influences (410). In all instances in this review the hormones have been defined as substances released by certain specific stimuli from the gastrointestinal mucosa. For some of the hormones, this differs from the definition given by the author who coined the name of the hormone, in that the original definition denoted an extract of mucosa which when injected produced effects mimicking a hormonal physiologic process. Inasmuch as there is seldom certainty that a tissue extract is the same as the endogenous circulating hormone, for clarity it would seem best to reserve the specific name for the endogenous hormone and to designate tissue extracts by qualified terms such as, for example, 'secretin extracts' or 'enterogastrone concentrates.' The nature of the difference between the endogenous hormone and the tissue extract may be of two kinds, *1*) the tissue extract may be derived from or related to the endogenous hormone, but in an altered chemical form or *2*) the tissue extract may be a non-specific substance mimicking the action of the endogenous hormone, but chemically and physiologically unrelated to it. Until these two kinds of lack of identity can be proven not to apply in any given case, it will serve the purpose of clarity to use separate terms to designate the endogenous hormone and the exogenous extract.

The term 'humoral mechanism' is used to designate a process which changes the function of an organ by the carrying to that organ of some blood-borne substance; this includes both hormonal and non-hormonal processes. Specifically, the term

humoral mechanism is used to designate a process which has been demonstrated to be independent of nervous connections between the site of stimulation and the effector site, and is, therefore, considered to be transmitted by a blood-borne substance, but in which the hormonal or non-hormonal nature of the blood-borne substance may be uncertain.

The term 'secretagogue' is used to designate that class of substances present in food or produced by the digestion of food, which excite secretion of digestive juice either by acting locally, or by being absorbed into the blood or lymph, or by causing a hormone to be formed.

Selye (410) divided endocrine organs on morphological grounds into *1*) purely endocrine glands, *2*) endo-exocrine glands and *3*) non-glandular endocrine organs. The glands producing the gastrointestinal hormones would fall into the group of endo-exocrine glands, since they secrete both an exocrine and an endocrine product. There is no information on the question of whether the same cells produce the exocrine and the endocrine components of the gastrointestinal secretions. In fact, beyond the knowledge that these hormones are formed in response to stimuli acting upon the gastrointestinal mucous membrane and that certain of them can be extracted from these mucous membranes, almost nothing is known about the exact cells which are responsible for their formation. This is a field of investigation which should be explored.

Relation to the Gastrointestinal Hormones to Other Endocrine Systems. One of the notable features of the endocrine organs other than the gastrointestinal glands is the interrelationship of their activities, producing a system of checks and balances. No such interrelationship has been discovered for the gastrointestinal hormones, either in regard to influences produced upon them by other endocrine organs or in regard to interrelationship among themselves. They are essentially an independent system, depending for their regulation and formation upon the appearance and withdrawal of stimuli in the form of food substances in the lumen of the alimentary canal. Although disease of endocrine organs such as, for example, the adrenal cortex may cause disturbances in the digestive system there is no evidence to suggest that this is due to an effect on gastrointestinal hormones.

As Ivy (221) has pointed out, the endocrine glands which embryologically are of mesodermal origin produce steroid hormones, whereas those of ectodermal and endodermal origin, including the gastrointestinal mucosa, produce protein or nitrogenous hormones.

The hormonally regulated processes in the gastrointestinal tract persist only so long as a stimulus is acting to cause release of the hormone. The latent period between the application of the stimulus and the hormonal response is usually a few minutes. Thus, the gastrointestinal hormones belong to the group of hormones with short latency and little persistence of action.

Special Problems Which Arise in the Study of Gastrointestinal Hormones. Most of the classical methods of endocrinology are poorly applicable to the study of the gastrointestinal hormones. For example, the most important general method for the study of other endocrine glands is to demonstrate that extirpation of the gland produces a characteristic deficiency state (410). In the case of the gastrointestinal

hormones, extirpation of the endocrine glands cannot be accomplished without simultaneous removal of the exocrine glands and the vital absorbing surface, leading to complicated changes which make it difficult to evaluate how much of the pathologic picture is due to loss of the endocrine function.

A common error in the interpretation of experiments designed to test whether a humoral mechanism exists is to conclude from a negative experiment that the postulated mechanism does not exist. Actually, all that can be said from a negative experiment is that "if the postulated humoral mechanism exists it cannot be demonstrated by the methods employed." In general the acute and chronic animal preparations used in the study of humoral mechanisms are relatively refractory when compared with the intact organ, and all but the most powerful stimuli may be ineffective. In acute experiments this is due to the inhibitory effect of operative trauma while in chronic experiments the decreased responsiveness resulting from interference with blood supply and from denervation occurs. Several instances can be cited of negative results in early experiments and positive results in later studies with more responsive preparations (for example, see the section on *Mechanical Stimuli* under GASTRIN).

Another difficulty arises from the fact that the target organs of the gastrointestinal hormones, the glands and muscles of the alimentary tract, are capable of responding to or being inhibited by a wide variety of substances including a number which are present in many tissue extracts and which cannot be considered to be specific hormonal products. For example, tissue extractives normally function as gastric secretagogues when meat is eaten and these secretagogues are capable of stimulating secretion after absorption into the blood. Thus, care must be taken to distinguish between secretagogues and hormones in tissue extracts. Since tissue extracts contain many pharmacologically active substances which are not hormonal in nature, it is of the greatest importance that the existence of a hormone be judged mainly on physiological evidence. Only when physiological experiments indicate the probable existence of a hormonal regulator should significance be attached to the isolation of an active substance from tissues. Until such experiments indicate that a hormone exists, tissue extracts which have an influence on the activity of organs cannot be regarded as hormones. For this reason the term 'hormone' will be used in this review to designate the substance liberated by physiologic stimuli, and it must be remembered that in no instance has proof been given that an active extract of the gland which produces the hormone contains the unaltered physiologic hormone.

Importance of Gastrointestinal Hormones in Body Economy. a) Dispensability. None of the gastrointestinal hormones are known to be essential to life, nor is there any disease which is now known to be associated with the overproduction or underproduction of these hormones. Several possible relationships between disease processes and the gastrointestinal hormones will be mentioned, however, in the discussion of the individual hormones.

b) Relation to nervous control of digestive organs. In general, every digestive function which is influenced by a gastrointestinal hormone also is regulated by nerves which act upon the same function. In such organs with both hormonal and nervous control, removal of the extrinsic nerves may depress the activity of the organ but

seldom is this sufficient to cause an impairment of digestion. Another generality that is worthy of note is the gradient of decreasing nervous and increasing hormonal control as one descends the gastrointestinal tract. Thus, the salivary glands are entirely under nervous control, the stomach has strong nervous and hormonal components of control, while the pancreas is mainly under hormonal control.

The synergistic relationship between nervous and hormonal stimuli and the influence of nerves upon the release of hormones will be discussed in later sections.

GASTRIN

Definition. Gastrin (Edkins, 1905, 95) is the hormone which is produced by the gastric mucosa in response to the action of mechanical and chemical stimuli and which stimulates hydrochloric acid secretion by the gastric glands.

Proof of Existence. The existence of a humoral mechanism for the gastric phase of gastric secretion was clearly indicated by the work of many early investigators (216). The final proof for the existence of such a mechanism was the demonstration by Ivy and Farrell (227) that the autotransplanted pouch of the fundic portion of the stomach secreted hydrochloric acid in response to the presence of a meal in the main stomach. This marked the beginning of the use of the technique of autotransplantation in the study of gastrointestinal hormones, a method which has since attained a position of great importance.

Once the existence of a humoral mechanism had been established the difficult task of determining whether it was hormonal in character remained. Two general types of stimuli, mechanical and chemical, act during the gastric phase. The evidence in regard to the hormonal mediation of these two kinds of stimuli will be considered separately.

a) Chemical stimuli. Three theoretical possibilities exist in regard to the mechanism of action of secretagogues in stimulating acid-secreting cells: *1)* direct stimulation by the chemical excitant in food either by local absorption or after absorption into the blood; *2)* stimulation by a degradation product of the secretagogue produced by digestion of the secretagogue or its alteration during absorption; and *3)* release of an acid-stimulating hormone by the action of the secretagogue on the mucosal cells.

The greatest obstacle to the clear-cut demonstration of a hormonal mechanism in the action of chemical stimuli for gastric secretion has been the fact that secretagogues which are effective locally in the stomach are also effective when given intravenously. Furthermore, no pure secretagogue of known chemical composition has been described. The most important secretagogues in food are the water soluble extractives of meats and cooked grains and the partial digestion products of protein. Undigested protein and amino acids are weak stimulants (216). The chemical nature of the stimulating agents in meat extracts and in the intermediate digestion products of protein is unknown, and the concentration of these agents in the blood cannot be measured.

From the study of secretagogues the following points of evidence supporting a hormonal mechanism have been derived: *1)* demonstration of the fact that secretagogues are more effective in stimulating secretion when they are placed in the stomach than when they are given intravenously and *2)* demonstration of the blocking action of procaine on the action of secretagogues on the gastric mucosa.

Kim and Ivy (253) showed that histamine-free liver extract was four times more effective when perfused through the stomach than when given slowly intravenously. This study was confirmed and extended by Butler *et al.* (56). These findings indicated that not all of the stimulatory action of the secretagogue could be accounted for on the basis of its action after absorption in an unaltered form into the blood stream. Kim and Ivy (253) found that incubation of a secretagogue (liver extract) with gastric juice did not enhance its stimulatory potency. This militates against, but does not rule out, the possibility that the secretagogue becomes altered into a more active substance during absorption.

Sawitsch and Zeliony (404) observed that application of certain substances to the pyloric mucosa stimulated secretion of acid by the fundic glands and that pretreatment of the pyloric pouch with cocaine prevented this effect. Recently Gregory and Ivy (178) made similar studies in dogs with a main stomach pouch and an autotransplanted fundic pouch. They found that application of procaine to the main stomach pouch prevented the secretory response in both pouches when secretagogue (liver extract) was perfused through the main stomach pouch. Since procainization of the transplanted pouch did not diminish its response to perfusion of secretagogue through the main stomach, the action of procaine could not be attributed to a paralysis of the parietal cells. Furthermore, Gregory and Ivy felt that it could not be accounted for on the basis of preventing the absorption of the secretagogue because the action of alcohol was not interfered with. It was concluded that the procaine acted by preventing the release of a hormone in response to action of the secretagogue.

The evidence derived from the study of secretagogues strongly suggests, but does not conclusively prove, that the humoral mechanism is hormonal.

b) Mechanical stimuli. Distension of the stomach is the only adequate mechanical stimulus for acid secretion. Lim, Ivy and McCarthy (301) showed that distension of a pyloric pouch with a balloon would induce acid secretion from a fistula of the remainder of the stomach, and distention of the main stomach would cause acid secretion by a 'denervated' fundic pouch (Heidenhain pouch with perivascular sympathetic fibers divided). The latter experiment was confirmed by Chang and Lim (60). These observations suggested that the distension stimulus was humorally transmitted. Since the classic work of Ivy and Farrell (227), however, it has generally been recognized that in order to prove that any particular mechanism of gastric secretion is humorally transmitted, it is necessary to demonstrate that mechanism by the use of the subcutaneously autotransplanted pouch. Unless the transplantation technique is used, the possibility of participation of extrinsic nerves cannot be eliminated with certainty, regardless of the thoroughness with which the denervation is done.

The first attempt to determine whether distention of the main stomach would evoke secretion in the transplanted fundic pouch was made by Gregory and Ivy (178). These workers observed no secretory response under the conditions of their experiments. Inasmuch as the studies cited above had clearly shown that the distention stimulus could be transmitted between two separated portions of the stomach, these negative results tended to indicate that this transmission depended upon nervous pathways which had not been completely severed in the earlier experiments but which were unquestionably interrupted in the transplantation operation. In

experiments of this type, however, a positive result is crucial whereas a negative result may be due to technical difficulties. For this reason, the problem has recently been reinvestigated by Grossman, Robertson and Ivy (188), who showed that distention of the pyloric portion could still stimulate acid secretion by the fundic glands when all nervous connections between these two parts of the stomach had been interrupted by transplantation of either. Inasmuch as the question of absorption of the stimulating agent does not arise in the consideration of the nature of the humoral agent for the distention stimulus, the conclusion that it is hormonal in nature would appear to be justified. This is strengthened by the specificity of the site of reception of the distention stimulus (see below).

The demonstration of the humoral transmission of the distention stimulus is the most crucial evidence which has thus far been adduced for the existence of a hormonal mechanism for stimulation of gastric acid secretion.

Mechanism of Release. a.) Site of formation. It has long been recognized that the stimuli for gastric secretion which act in the stomach (distention and secretagogues) are more effective when acting in the pyloric than in the fundic region (216). This indicates that the predominant site of formation of gastrin is the pyloric mucous membrane. Thus, it has been clearly demonstrated that pylorectomy markedly reduces the response of a total stomach pouch to distention and secretagogues (178, 252, 476). It should be emphasized that these same experiments showed that the isolated, denervated fundic pouch does give a small secretory response to secretagogues and distention, indicating that gastrin formation is probably not confined entirely to the pyloric region. Special mention is made of this point because some workers in this field have made statements which indicate that they believe that the pyloric mucosa is the only portion of the stomach capable of responding to the stimuli for the gastric phase of acid secretion.

While these results clearly establish the importance of the pyloric part in the initiation of the stimuli for the gastric phase of gastric secretion, they do not enable us to evaluate the relative contribution of the pyloric component in the total gastric secretory response to a meal. The reports on the effect of pylorectomy upon the secretory response of the remainder of the stomach, or of a Pavlov pouch, to a meal provide some information on this question. In interpreting the results of these experiments, however, it should be remembered that pylorectomy produces other changes in gastric function, such as increased rate of emptying, and alteration of the level at which chyme enters the intestine, so that changes which occur cannot, without further analysis, be attributed simply to the removal of gastrin production by the pyloric mucosa.

Does resection of the pyloric part of the stomach reduce the acidity of the gastric contents in response to a test meal? All who have investigated this problem in dogs (109, 298, 310, 364, 411, 465, 476) agree that a moderate to marked depression to acidity of the gastric contents occur. The only dissenting opinion is that of Priestley and Mann (366) who observed no change in acid concentration of the gastric contents following resection of the pars pylorica. The last-mentioned investigators compared only the maximal response to the test meal pre- and post-operatively, giving no consideration to average values. Those workers who made a special study

of the early and late effects of the operation all agree that there is a tendency for the acid values to return to the pre-operative control level within a relatively short period of time (several weeks to several months).

The secretory response of isolated fundic pouches before and after pylorectomy of the main stomach has also been studied by a number of investigators (102, 411, 418, 425, 465). All found that pylorectomy produced depression of the secretory response in a Pavlov pouch. The only workers who failed to observe a consistent depression were Portis and Portis (364). Woodward and his co-workers (478) recently studied the total 24-hour acid output of Pavlov pouches before and after pylorectomy and they too found significant depression. As in the studies on the intact stomach, those investigators who did repeated studies over a number of months (102, 411, 479) found a distinct tendency for a return to normal secretory levels.

Klein (262) found that the response of a subcutaneously transplanted fundic pouch to a meal was abolished by resection of the antrum of the main stomach. This cannot be considered to be a typical result because Gregory and Ivy (178) found that removal of the entire stomach from the alimentary pathway did not abolish the response of a transplanted pouch to a meal. The 24-hour secretory response of Heidenhain pouch dogs was not significantly affected by resection of the pyloric portion of the main stomach according to Grindlay (179).

In general these results indicate that resection of the pyloric portion of the stomach usually produces a moderate depression in the secretion of acid in response to a meal and that recovery tends to occur in a few months.

Some surgeons (14, 464) on the basis of their experience with the surgical treatment of peptic ulcer, believe that even when the pyloric portion of the stomach has been excluded from the alimentary pathway it still exerts an excitosecretory effect on the fundic glands. There are a considerable number of case records in which the removal of the excluded pyloric antrum is said to have been followed by healing of post-operative jejunal ulcers (469). This suggests that, at least in the ulcer patient, the pyloric mucosa (even when the food is diverted away from it) can still release stimuli which activate the fundic glands. The few experiments designed to test the effect of the pyloric portion of the stomach, isolated from the alimentary canal in the form of a pouch, have indicated that its removal had no effect on the secretory response of the fundic glands to a meal (465). A suggestive piece of evidence on this question comes from the study of Chang and Lim (60) who found that removal of an isolated pyloric pouch abolished the secretory response of a fundic pouch to such mechanical stimuli as the feeding of bones. Obviously this question of the effect on gastric secretion of the isolated pyloric portion of the stomach deserves further study.

The presence of secretagogues in the small intestine causes a transplanted fundic pouch to secrete acid but distention of the intestine (unlike the stomach) does not stimulate acid secretion (178). That a difference between the stomach and intestine may exist in regard to the mechanism of action of secretagogues at these sites is suggested by several facts. First, the latent period between the introduction of the secretagogue and the onset of gastric secretion is distinctly longer in the intestine than in the stomach (253). Second, certain secretagogues act in the intestine but not in the stomach (231). No evidence on the effect of procainization of the intestinal

mucosa or on the relative effectiveness of secretagogues perfused through the intestine and given intravenously are available. There is, therefore, at present no physiologic evidence on which to judge whether the humoral mechanism is hormonal. The evidence from the studies on the extraction of gastrin preparations from the intestinal mucosa will be considered in another section.

b) Evidence for participation of a nervous factor in the release of gastrin. Recent studies have revealed that the nervous and humoral mechanisms of the control of the digestive system are not completely independent, but that each has important influences upon the other. Thomas (435) has emphasized this viewpoint. These interrelations will be discussed now in regard to gastric secretion and in later sections, where appropriate, for other functions.

In the dog a moderate dose of atropine (1 mg. subcutaneously) will abolish the response to secretagogues or distention acting in the stomach (301). Inasmuch as atropine does not abolish the secretory response to the injection of any of the gastrin extracts which have thus far been made (149, 261, 275) (also see below), the suggestion has arisen that atropine acts by preventing the secretagogue or distention from releasing gastrin (149, 219).

To the information given above concerning the effect of procaine should be added the fact that application of this drug to the gastric mucosa prevents the stimulation produced by distention. Since procaine does not inhibit the response of the parietal cells to gastrin, it has been assumed to act by preventing the release of gastrin (178).

These observations on the effects of drugs which are known to inhibit certain kinds of nervous activity have led to the suggestion that a nervous mechanism may be concerned in controlling the release of gastrin (219). In its simplest form the theory for such a mechanism would envisage a nerve cell, its cell body probably located in the submucosal plexus, with a receptor mechanism capable of being anesthetized by procainization of the mucosa, and with an efferent limb playing upon the cell which produces gastrin. The action of this efferent limb can be blocked by atropine and it is therefore probably cholinergic. In support of this theory is the recent demonstration that acetylcholine perfused onto the pyloric mucosa, is a strong stimulant for the release of gastrin (382).

Uvnäs (446) and Kahlson (243) have suggested that there is a more obvious relation between the nervous system and the release of gastrin. These investigators believe that stimulation of the vagus nerves to the stomach results in the release of gastrin and that neither the vagal nor the gastrin mechanism can operate to its full extent in the absence of the other. The evidence for this view consists of experiments on anesthetized cats and dogs in which it was shown that when the pyloric region was inactivated by ligation, removal of the mucosa, cocainization of the mucosa, or resection of the entire pars pylorica, the amount of acid secreted in response to electrical stimulation of the vagi was reduced. The view that vagal stimuli and gastrin can act together in stimulating gastric secretion is certainly in accord with observations, in the sense that any two different stimuli can act together additively. However, the view that either of these two mechanisms for secretion regularly participates in producing a response to the other is open to serious question. That the gas-

trin mechanism can operate in the complete absence of the vagus nerve is obvious from the many studies on the effects of secretagogues and distention in vagally denervated stomach pouches (201). Furthermore, there is evidence to indicate that strong physiologic stimulation of the vagal secretory nerves to the stomach does not lead to the release of gastrin. For example, in a dog with a Heidenhain pouch (vagally denervated), Jemerin and co-workers (240) showed that insulin hypoglycemia or sham feeding produced strong vagal stimulation of the main stomach with high acid production, yet no secretory response occurred in the pouch. If the vagal stimulation were leading to release of gastrin from the pyloric portion of the main stomach we should expect it to circulate in the blood stream and to cause stimulation of the pouch. Unless the Heidenhain pouches studied by Jemerin were too refractory to respond to gastrin, his experiments would seem to constitute crucial evidence in disproof of the possibility that vagal stimuli release gastrin.

Babkin and co-workers (24, 25) attempted to repeat the experiments of Uvnäs but were unable to demonstrate that extirpation of the pyloric mucosa interfered with the secretory response to electrical stimulation of the vagi except in those experiments where trauma and vascular impairment obviously played a role.

Accurate studies are not available on the effect of vagotomy on the secretory response to stimuli which act by releasing gastrin. As mentioned above, we know that vagotomy does not abolish these responses, but it may impair them. In the dog the total secretory response to a mixed meal is significantly depressed by vagotomy (454), but there is a strong tendency for recovery to occur. In the human the secretory response to a meal is also depressed by vagotomy (331). This decrease in response to a meal after vagotomy cannot be attributed to a specific relationship between the gastrin and vagal mechanisms, but appears to be due to a general decrease in the responsiveness of the parietal cells to any kind of stimulus, including histamine (19, 424), which occurs after vagotomy.

There is some evidence to indicate that when cholinergic and hormonal stimuli are acting simultaneously, their combined effect is not merely additive but may be synergistic (381).

Tissue Extracts with Gastrin Activity. a) Edkins' gastrin. One frequently sees the statement that a certain piece of evidence does or does not, as the case may be, support 'Edkins' hypothesis.' Because of the prevalence of this expression it is desirable to reexamine the work of Edkins to determine just what his hypothesis was and to evaluate it in the light of present day knowledge. Edkins, working by analogy with the then recent discovery of secretin by Bayliss and Starling, proposed that substances which stimulate gastric secretion by their presence in the stomach act by causing the release of a hormone, 'gastrin' or 'gastric secretin,' from the pyloric mucosa. Edkins offered two types of evidence in support of this hypothesis. The first was that acid extracts of pyloric mucosa stimulated gastric secretion. This evidence will be examined in the following paragraph. The second type of evidence consisted of an attempt to demonstrate that the presence of food substances in the pyloric portion of the stomach induced acid secretion by the fundic glands (97). These experiments were performed upon anesthetized animals and when they were repeated by Ivy and Whitlow (239) it was shown that the 'responses' observed by Edkins

were all within the limits of spontaneous variation of unstimulated dogs. Although the experiments of Edkins were inadequate to establish the point, subsequent studies on chronic gastric pouch animals clearly established that chemical and mechanical stimuli acting on the pyloric mucosa can stimulate the fundic glands, as has been discussed above. We may conclude then that the hypothesis that Edkins, on the basis of his tissue extract work, set forth concerning the special role of the pyloric mucosa as a site for gastrin production, has been amply substantiated by subsequent physiologic experimentation, although his own physiologic evidence was inadequate and his tissue extract work is still disputed.

Edkins (96) observed that acid extracts of the pyloric mucosa stimulated acid secretion when injected *intravenously* into anesthetized cats. Lim (300), the only investigator who repeated Edkins' experiments on tissue extracts essentially as Edkins had done them (intravenous injection in anesthetized animals of simple acid extracts), confirmed Edkins' findings. Other investigators modified Edkins' procedure by *1*) using different extraction procedures and/or *2*) injecting their extracts *subcutaneously*. Such subcutaneously effective extracts can be prepared from both pyloric and fundic mucosa as well as from many other tissues (42). A vast amount of investigation (216) on these subcutaneously effective extracts culminated in the isolation of histamine as the crystalline picrate from them by Sacks *et al.* (384). That histamine was the sole active ingredient in the subcutaneously effective extracts which they studied was demonstrated in two ways (384): *1*) the vasodepressor and gastric stimulatory activities showed direct parallelism through all stages of purification and *2*) histaminase destroyed both the vasodepressor and secretory stimulating activities. We shall return to the question of the possible physiological role of histamine in controlling gastric secretion.

It is unquestionable that histamine was present in the extracts studied by Edkins and by Lim, but that it accounted entirely for the stimulation they observed is open to question because there was no parallelism between vasodepressor and secretory activity of the extracts. For example, both investigators found fundic extracts to be ineffective in inducing acid secretion despite the fact that they produced as much fall in blood pressure as did the pyloric extracts. Neither Edkins nor Lim did experiments which were quantitative enough to establish beyond doubt this lack of parallelism between secretory stimulation and vasodepression. In the light of recent investigation, however, which will now be presented, these observations acquire new significance.

b) *Komarov's gastrin.* Komarov (273) extracted from the pyloric mucous membrane a gastric secretory excitant which was apparently free of histamine. In this and subsequent studies (274, 275), methods of extraction were used which were designed to purify the protein constituents and to free them of nitrogenous bases of small molecular size, such as choline and histamine. This approach was adopted to test the possibility that concentration of gastrin might be accomplished by the same general type of procedure which had been successfully used in the purification of secretin. Again, working by analogy to secretin, where only the intravenous route is highly effective, Komarov injected his gastrin preparation intravenously; in this respect, he differed from most of the previous investigators of gastrin since the early

studies of Edkins and Lim. The gastrin preparations of Komarov have many similarities to crude secretin such as precipitability by trichloracetic acid or by saturation with sodium chloride, solubility in acidified 1:4 mixtures of water and methyl alcohol, ethyl alcohol or acetone, and insolubility in pure organic solvents. Indeed, Komarov's cruder preparations contained secretin in significant concentration but this could be removed by further purification and the final product had no effect upon any digestive glands other than the stomach, nor did it lower the blood pressure. These gastrin preparations were found to be effective in anesthetized dogs and cats and unanesthetized dogs with gastric fistulas.

Doses of 200 mg. of the purified extract given intravenously in gastric fistula dogs induced a secretory response as high as 20 cc. of gastric juice in the ensuing hour, with a rise in the free acid concentration to as high as 110 mN, and a fall in pepsin concentration to very low levels. Intramuscular injection of these gastrin extracts was stated to produce a small secretory response whereas subcutaneous administration was totally ineffective. Atropine in doses as high as 2 mg. per kg. of body weight failed to abolish the response.

Study of the distribution of gastrin revealed that the most potent extracts are obtainable from the pyloric mucosa; the duodenal mucosal extracts were much less active, and the jejunal mucosa, fundic mucosa of the stomach and liver gave inactive extracts.

Uvnäs (446) has used Komarov's method of preparing gastrin and has made some modifications. The results of Uvnäs confirm those of Komarov in most respects. Uvnäs' (449) most potent extract, when injected intravenously at a rate of 20 μg per kg. body weight per minute for 15 minutes into anesthetized cats, produced a secretory response of 4 cc. of gastric juice with high acid concentration. Uvnäs stated that 10 to 15 μg. of histamine diphosphate per kg. body weight per minute must be given to produce a comparable response. Potent extracts were prepared from the pyloric mucosa of dogs, cats, pigs (447) and humans (450). Fundic and duodenal mucosa, liver, spleen and skeletal muscle gave inactive extracts (449). Certain active pyloric extracts, after a period of stimulation, caused a depression in the responsiveness to other extracts or to histamine, suggesting the presence of a secretory depressant in the extracts (449). The purer extracts did not stimulate pancreatic, biliary or salivary secretion, and had no effect on blood sugar, gastric motility or blood pressure (334). The histamine content of active extracts was usually too small to be detected and never exceeded 30 μg. per liter (447). Incubation with histaminase failed to reduce the effectiveness of potent extracts (30). Uvnäs (448) found that the active principle resisted heating in N/10 HCl at 100° C. but was destroyed when heated to this temperature with N/10 NaOH. Commercial pepsin, pancreatic juice activated by duodenal juice, and ultraviolet light inactivated the extracts. The active principle was dialyzable through cellophane and was precipitated by trichloracetic acid, metaphosphoric acid, saturated solutions of sodium chloride, or acetone. Both Komarov (275) and Uvnäs (449) stated that the pepsin concentration of the gastric juice falls to extremely low levels under stimulation with these extracts.

Harper (220) applied Mellanby's method for preparation of secretin to hog pyloric mucosa as a means of extracting gastrin. Pyloric mucosal extracts were most

active, upper small intestinal extracts less so, and extracts from the mucosa of the body of the stomach or lower part of the intestine were inactive. Harper found that the active material did not dialyze through cellophane, was destroyed by pepsin and had no depressor action on the blood pressure of the cat. Using a method for the preparation of secretin, Friedman and King (127) obtained active gastrin extracts from the pyloric mucosa of dogs.

Hanson and Grossman (197) have prepared many extracts of pyloric mucosa according to the secretin procedure of Ivy *et al.* (232). None of these stimulated gastric secretion when administered intravenously. In their hands the method of Komarov gave a few active extracts but more inactive ones. When tested in unanesthetized dogs with gastric pouches a single extract sometimes stimulated and at other times was ineffective.

The important work of Komarov may mark a turning point in the history of gastrin extraction. When further confirmatory reports from a number of different laboratories appear, this work will be on firm footing.

c) Histamine. It is not known with certainty whether histamine plays a physiological role in any normal process in the body, including gastric secretion. There are two theories as to how histamine may be involved in the normal secretory process. The first proposes that histamine may be gastrin and thus may be the circulating humoral agent for the gastric phase of gastric secretion. The second theory proposes that histamine acts as a local chemical agent released in close relation to the parietal cell in response to all kinds of stimuli which are capable of stimulating acid secretion. Although a final answer cannot be given, much important evidence bearing on this question of a physiological role for histamine in gastric secretion is at hand.

Histamine is known to be present in many body tissues including blood, liver, lung and skin. Large amounts are present in the mucosa of the stomach and of the intestine, whereas the muscular coats of these organs contain little histamine. As noted above, Sacks *et al.* (384) isolated crystalline histamine picrate from hog's pyloric mucosa. Gavin *et al.* (140) showed that large amounts of histamine activity can be extracted from both the pyloric and fundic mucosa of the dog, a finding which was confirmed for the human stomach by Trach *et al.* (445), and for the cat by Emmelin and Kahlson (101). Histaminase is present in high concentration in the intestinal mucosa but is stated to be absent from gastric mucosa (35). This observation requires confirmation.

Histamine is the most potent stimulant known for gastric acid secretion, although some choline esters such as mecholyl may be equally potent on a molar basis (155). The parietal cells appear to be more sensitive to histamine than any other cells in the body. Recently Hanson and co-workers (199) have studied the rate of histamine administration required to produce a gastric acid response in unanesthetized man and dogs. The average minimal effective dose was 0.04 μg. of histamine base/kg/minute for dog, and 0.004 μg/kg/minute for man. In the studies on man it was observed that rates of histamine administration which were only slightly higher than those required to produce a gastric secretory response frequently produced flush or headache. This is a significant observation in that it indicates that if histamine were a humoral agent for gastric secretion in response to a meal the systemic effects that

accompany the presence of this amount of histamine in the circulation would be expected to occur.

Emmelin and Kahlson (101) showed that the minimal rate of histamine administration required to produce a detectable rise in plasma histamine level by currently available methods of assay is 2 to 5 μg/kg/minute. When this figure is compared with the dose of histamine required to stimulate secretion of acid by the dog's stomach maximally, it is apparent that doses of histamine too small to cause a detectable rise in plasma histamine level can produce marked stimulation of gastric secretion. The crucial evidence for the participation of histamine as a humoral agent in the acid response to a meal will rest on the ability to detect a rise in plasma histamine of the systemic blood during digestion; but, a rise large enough to produce the entire secretory response may occur without being detectible by currently available methods. The parietal cell is more sensitive to histamine than are our methods for assay of blood histamine.

The studies which have been made on blood histamine levels during digestion have all been done on whole blood (281, 316, 430). Since most of the histamine found in whole blood is derived from the blood cells where it is found in inactive form (65, 100), studies on whole blood are not likely to be helpful. On the other hand, studies on plasma histamine levels are attended with serious technical difficulties because tissue trauma, clotting, agitation of blood etc. all cause a rise in plasma histamine. Nevertheless, such studies should be attempted. Emmelin (100) has set forth the view that a small amount of histamine is normally present in plasma in physiologically active form. He proposes that the responsive cells become adapted to this constant level of histamine and that responses take place only when an increment in plasma histamine level occurs.

Komarov (272), in partitioning the non-protein nitrogen fractions of gastric juice, found that the 'arginine' fraction exerted a histamine-like effect. Brown and Smith (50) extracted from human gastric juice a substance with properties like histamine. MacIntosh (316) applied the Barsoum-Gaddum method and found histamine-like activity to be present in gastric juice obtained from dogs by sham feeding, subcutaneous injection of histamine and electrical stimulation of the vagi. MacIntosh suggested that the concentration of histamine in the juice varied with the rate of secretion, high secretory rates being associated with high histamine concentration of the juice. Emmelin and Kahlson (101) confirmed the presence of a histamine-like substance in the gastric juice of cats and dogs, secreted in response to a variety of stimuli, but they found no correlation between histamine concentration of the juice and rate of secretion. Code *et al.* (67) in a recent careful study also found histamine-like activity to be present in most of the samples of gastric juice collected from dogs with gastric pouches. They too used a wide variety of physiologic and pharmacologic stimuli and found histamine-like activity in most of the samples of juice secreted in response to each of the stimuli. Some samples of gastric juice, including some secreted in response to subcutaneous injection of histamine, had too little histamine to detect by the method used (less than 2.5 μg/liter). The highest values occurred in the juice secreted in response to mecholyl. In contrast to MacIntosh (316), Code *et al.* (67) found that, in general, high concentrations of histamine activity

tended to occur in juice in which the concentration of HCl was comparatively low and vice versa.

All of these investigators found that histamine may occur in higher concentration in the gastric juice than in the plasma. For this reason and because the gastric mucosa has much histamine and no histaminase, the histamine in gastric juice is believed to arise from the gastric mucosa. Apparently none of these investigators studied the histamine concentration of non-acid gastric juice. It would also be of interest to determine what part of the histamine in gastric juice may arise from the disintegration of shed surface epithelial cells.

Babkin (21, 22) is the originator of the theory that histamine may be the final common local chemical agent for gastric acid secretion regardless of what stimulus is acting to initiate the secretion. He believes that under normal circumstances the source of this histamine is the gastric mucosa, from which it is released in close proximity to the parietal cell. The histamine thus released is believed to pass through the parietal cell and to be excreted into the gastric juice. The theory requires that the histamine be excreted quantitatively by the parietal cell since none of it can be detected in the portal blood by biologic assay and no systemic symptoms of histamine release occur. One crucial point of evidence in regard to this theory will be to determine whether the parietal cell is permeable to histamine by learning whether plasma histamine appears in the gastric juice when gastric secretion is being stimulated by administration of histamine. Emmelin and Kahlson (101) performed experiments correlating the rate of histamine administration with the concentration of histamine in the gastric juice. They interpreted the results of these experiments as indicating that the blood level of histamine is reflected in the gastric juice level; however, they use enormous rates of histamine administration which caused marked depression of gastric secretion and they did not determine the plasma histamine levels.

An important step could be made in determining the role of histamine in the normal control of gastric secretion if there were a drug which would specifically block the action of histamine on the parietal cells. No such drug is known to exist. The recently discovered antihistaminic drugs fail to reduce the gastric secretory response to histamine (400, 305). It would appear that these drugs lack the ability to attach to and block the receptor substance for histamine in the parietal cell.

Until recently, the available preparations of histaminase were too toxic to administer in doses large enough to produce effects *in vivo*. Grossman and Robertson (187) have studied a hog kidney histaminase preparation of low toxicity and have found it to be capable of greatly reducing or abolishing the gastric secretory response to histamine, as well as to food and to other drugs. If it were certain that the depressing power of this kidney extract was due entirely to its histaminase content, these findings would constitute a strong argument in favor of the view that histamine is the final common local chemical mediator for all stimulants of gastric acid secretion. Because of the crude nature of this histaminase preparation, judgment must be deferred.

Histamine has been shown to be released in the body under various pathologic conditions such as anaphylactic and anaphylactoid shock (89). Under these circum-

stances the systemic effects such as fall in blood pressure are usually so profound that gastric secretion is prevented from occurring. However, there are some instances of milder degrees of histamine release in which gastric secretion may occur. For example, Kalk (244) has demonstrated that gastric secretion may be stimulated by stroking the skin of persons with dermographism.

There are a number of agents which produce injury of the mucosa when they are perfused through the small intestine. HCl is such an agent. When such damage occurs a stimulation of gastric secretion is usually observed (235). Since injury of tissues is known to be capable of causing histamine release, it has been suggested that this is the mechanism by which those agents act (219). In dogs with the Mann-Williamson operation for the experimental production of peptic ulcer an abnormally high basal level of secretion occurs and, like histamine-stimulated secretion, it is not completely abolished by administration of 1 mg. of atropine sulfate. It has been proposed that the hypersecretion is caused by the release of histamine by the jejunal mucosa injured by acid (225).

It is generally taught that the gastric juice of the gastric phase of gastric secretion has a moderate pepsin content, lower than that of the cephalic phase but higher than that secreted in response to histamine (22). Exact data on this question are difficult to gather because the lack of standards in pepsin determinations makes it impossible to compare the results of various laboratories in terms of absolute units. Grossman *et al.* (191) studied the pepsin content of gastric juice secreted by transplanted fundic pouches in response to perfusion of the main stomach with liver extract, and found that it was low like that obtained with histamine and unlike that secreted in response to pilocarpine. The doses of drugs were selected so as to give a rate of secretion comparable to that obtained by perfusion of the secretagogue. The evidence that the response of the transplanted pouch in this experiment involves a hormonal mechanism has already been given. Assuming then, that this represents a physiologic release of gastrin, we may say that gastrin is not a strong stimulant for pepsin secretion.

Both histamine and Komarov's gastrin extract produce a gastric juice of very low pepsin content (275). In regard to histamine there is a dispute as to whether it stimulates pepsin secretion at all (36, 46, 53). Until standardized methods for pepsin determination are adopted so that we can speak of pepsin in terms of a reproducible unit, this dispute is not likely to be settled. Meanwhile all are agreed that the juice secreted in response to histamine has a low pepsin content when compared with that produced in response to parasympathomimetic drugs (22).

SECRETIN

Definition. Secretin (Bayliss and Starling, 1902, 32) is the hormone which is released when the upper intestinal mucosa is exposed to certain chemical substances, and which stimulates the secretion of pancreatic juice. Several reviews devoted to secretin are available (59, 285, 426).

Proof of Existence. By 1930 the existence of secretin had been rigorously proven by a number of different investigators using a variety of methods including transplantation, cross circulation, and isolation of highly potent and specific secretin

concentrates. This evidence was thoroughly reviewed by Ivy (216). Since that time several workers have added still further observations on homo- and autotransplantation of the pancreas into the neck by vessel anastomosis (141, 210).

Mechanism of Release. a) Sites of formation. Pavlov (354) had shown that acid stimulated pancreatic secretion only when it is placed in the upper portion of the small intestine and failed to do so when confined to the stomach or rectum. This has been repeatedly confirmed, but very little further work has been done on the relative effectiveness of various other known stimulants of pancreatic secretion acting at various levels of the gastrointestinal tract. No one has succeeded in demonstrating stimulation of pancreatic secretion when various substances are placed in contact with the gastric mucosa (216) although the gastric mucosa (both pyloric and fundic) is the only tissue other than the small intestinal mucosa from which *vasodilatin-free* secretin can be extracted (92, 275, 334). The concentration of secretin in gastric mucosa is decidedly lower than in the mucosa of the small intestine.

Although it had been clearly demonstrated that the 'secretins' extractable from a wide variety of plant and animal tissues owed their activity to histamine and other vasodilatins and were therefore not the same as the potent, specific secretin extractable from gastrointestinal mucosa which can be entirely freed of vasodilatin (232), as late as 1938 we find a paper by Krimberg (282) on a secretin extracted from muscle upon which no data on vasodilatin content are given.

The suggestion of Wermel and Kacharova (471) that the basal granular cells (argentaffine) of the mucosa of the small intestine are the site of secretin formation is based entirely on histological evidence and will require physiologic support. It does not take account of the fact that these cells are plentiful in the vermiform appendix where no secretin has ever been demonstrated.

b) Prosecretin. As Scott and Still (407) clearly showed, the secretin in the intestinal mucosa is found in a form which is more readily extracted with acid than with neutral solvents. This is probably due to the adsorption of the secretin onto colloidal components of the mucosa and not to the conversion of a 'prosecretin' into secretin by the acid as was originally suggested by Bayliss and Starling (32).

c) Stimulation of secretin release by HCl. The stimulant for pancreatic secretion which has been most extensively studied is hydrochloric acid. Thomas and Crider (438) have determined the *p*H value required to be present in the duodenum to produce stimulation of pancreatic secretion in unanesthetized dogs. When strong buffers were used to maintain the *p*H of the intestinal contents relatively constant, a *p*H of 4 to 5 was sufficiently acid to stimulate. Thomas (432) has shown that the median value for *p*H of the duodenal contents following a meat meal is about 4.0, indicating that under physiologic conditions the duodenal contents become sufficiently acidic to stimulate pancreatic secretion. However, other studies from Thomas' laboratory (357)have shown that the pattern of volume response of pancreatic secretion following a meal of meat cannot be accounted for solely on the basis of fluctuations in *p*H of the duodenal contents, suggesting that stimuli other than acid are also important under these physiologic conditions.

d) Stimulation of secretin release by substances other than HCl. There is implicit in the reasoning found in many papers on pancreatic secretion the notion that HCl

is the only stimulant for secretin release and conversely that stimulation produced by agents other than acid is not due to secretin (434). Gray *et al.* (156) found that products of protein, carbohydrate, and fat digestion did not stimulate pancreatic secretion upon intravenous injection unless a fall in blood pressure occurred. This indicates that absorption into the blood stream of secretagogues probably does not play an important role in stimulation of pancreatic secretion. On the other hand, it is known that starch, fat or protein will stimulate secretion from a transplanted pancreas (216). Since, therefore, these substances do act, at least in part, by a humoral mechanism and probably do not act by being absorbed, the most important possibility is that they cause the release of secretin. The other possibility is that these substances act indirectly by causing bile or gastric juice to enter the duodenum. Earlier evidence had suggested that peptone acted by stimulating gastric secretion (216), but Thomas and Crider (439) have provided evidence to show that products of protein digestion do not depend upon entry of bile or gastric juice into the duodenum for their ability to stimulate pancreatic secretion. The final proof that these foodstuffs cause secretin to be released will probably wait upon a method for determining the blood level of secretin.

Crider and Thomas (71) expressed the view that substances such as peptones excite pancreatic secretion by means of local reflex arcs between the duodenum and pancreas. This view does not take into account the fact that a meat meal will stimulate the transplanted pancreas to secrete, proving that this mechanism is at least in part independent of nervous connections between the duodenum and pancreas.

The only substances which have been shown to be capable of causing the transplanted pancreas to secrete when they are perfused through a Thiry loop of small intestine are HCl, digested fat and bile. Neutral fat was ineffective. Other substances, such as peptone, have not been tested (216). If other substances should be shown to be effective by this method it would remain to be proven that they do not act by causing bile or gastric juice to enter the duodenum. On the other hand, substances found to be ineffective by this method cannot be dismissed as possible excitants of secretin formation because of the limitation that must be placed upon the interpretation of negative experiments (see introduction).

e) The question of the participation of a nervous mechanism in the release of secretin. Atropine in moderate doses does not markedly reduce the response to secretin injected intravenously or to acid placed in the duodenum. It may produce some reduction in the response to these stimuli as well as to soap placed in the duodenum (441). Unlike the gastric secretory response to a meal, the pancreatic secretory response is not abolished by atropine, although it may be reduced. Atropine may reduce or abolish the response to a stimulus being perfused through a Thiry loop of intestine but have little or no effect on the same stimulus acting in the duodenum *in situ*. This is true for acid (228) and for peptone (441). These differences may be due solely to quantitative relationships between the strength of the stimulus and the dose of atropine, i.e. the substances acting in the isolated loops may be weaker stimulants and therefore more easily inhibited.

The partial inhibitory effects of atropine which have been noted may be due *1*) to the blocking of acetylcholine produced in subthreshold quantities by the tonic ac-

tivity of the postganglionic vagal fibers in the pancreas; *2*) to a direct blocking effect of atropine upon secretin which may occur in certain dose ranges; *3*) to partial interference with secretin release; or *4*) to the blocking of secretory reflexes over extrinsic pathways or over local nervous networks. There is no clear evidence in support of any of these theoretical possibilities.

The effect of perfusion of the upper intestine with local anesthetic agents on the effectiveness of pancreatic secretagogues has not been studied.

That many different types of stimuli can still excite pancreatic secretion after vagal connections have been severed is obvious from the studies on the transplanted pancreas. Earlier studies showed that vagotomy did not abolish the response to secretin (377, *inter alia*). A quantitative study of the effects of vagotomy, however, has only recently been made. Crider and Thomas (71) found that immediately after vagotomy the response to peptone was markedly depressed but that partial recovery of the volume response occurred in a few days. The response to HCl was moderately reduced while that to soap was approximately the same after vagotomy.

The effects of the administration of atropine and of vagotomy on pancreatic secretion do not provide direct evidence for either the participation of nerves in secretin release or the existence of reflexes for pancreatic stimulation, nor do they rule out these possibilities. The effect of these procedures could be explained by assuming that the postganglionic vagal fibers constantly form acetylcholine in amounts too small to produce stimulation alone but sufficient to potentiate the response to secretin. According to this hypothesis, atropine blocks the action of this acetylcholine, and cutting of the preganglionic vagal trunks temporarily reduces the formation of acetylcholine by the postganglionic fibers.

Preparation and Chemical Properties of Secretin. a) Units of potency. Great difficulty has been encountered in making accurate comparison of the potency of various purified preparations of secretin because of the lack, until recently, of an accurate unit for the expression of potency. The cat unit proposed by Wilander and Ågren (474) is totally unsatisfactory. It depends upon the titration of the alkali collected upon a piece of filter paper placed in the duodenum opposite the pancreatic duct. Aside from the technical inadequacies, it fails to take into account the variability in response from animal to animal. The Ivy dog unit (232) attempts to overcome this latter shortcoming by defining the secretin unit as the amount of material which upon intravenous injection will, in the average dog, cause an increase in pancreatic secretion amounting to 10 drops (0.4 cc.) in 10 minutes. This unit has the advantage that it is self-defined, requiring no reference standard for comparison. It has the great handicap of being tedious to use, requiring determinations on many dogs in order to establish the potency of a single preparation. In recent years in the field of biological assays the principle has become firmly established of using a reference standard preparation and performing parallel tests on both the reference standard and the unknown sample (41). Burn and Holton (54) have recently suggested that this principle be applied to the assay of secretin using acetone-dried intestinal mucosa as a reference standard and using the ratio of the slopes of the dose response curves as a measure of potency. Gershbein *et al.* (146) have made a similar suggestion but they propose that SI (a crude extract of intestinal mucosa prepared by a standardized

technic) (232) be used as the reference standard and that the unitage of potency be expressed as the ratio of weight of the unknown to weight of the standard required to produce the same responses at any submaximal rate of stimulation. The success which these assay methods will enjoy will depend mainly upon the reproducibility, stability and particularly upon the general availability of the reference standards.

b) ***Highly purified preparations.*** Two groups of investigators have made crystalline preparations of secretin. In both instances the crystalline product was prepared as a compound with picrolonic acid; there are, however, important differences between these two materials. The water-insoluble crystalline secretin picrolonate of both Hammarsten and Ågren (1, 5, 13, 194) and Greengard and Ivy (170) can be converted to water soluble simple salts such as chlorides or phosphates, but these are invariably amorphous.

The crystalline secretin picrolonate prepared by Greengard and Ivy (170) utilized the Weaver, Luckhardt and Koch (468) procedure of dilute acid extraction of hog intestinal mucosa followed by precipitation by saturation with sodium chloride. This precipitate is designated 'A precipitate' by Ivy and co-workers and serves as an excellent starting material for the purification of secretin, pancreozymin, cholecystokinin, duocrinin, and enterogastrone. This salt precipitate was extracted with 70 per cent alcohol, the alcohol was removed by distillation, and the activity was precipitated by the addition of trichloracetic acid to give a final concentration of 5 per cent. This is the 'SI' precipitate of Ivy and co-workers (232); it is free of vasodilatin and contains secretin, pancreozymin, cholecystokinin and duocrinin. Greengard and Ivy (170) fractionated SI by dissolving in acidified 80 per cent acetone and adding aniline to the point of complete precipitation. The supernatant fluid was freed from acetone and aniline by vacuum distillation, evaporated to dryness, extracted with methyl alcohol and precipitated with an excess of ether. The resulting precipitate was dissolved in water and extracted with n-butyl alcohol. The addition of picrolonic acid resulted in a crystalline precipitate which appeared in the form of clusters of yellow needles when viewed under the microscope. X-ray diffraction revealed that it was uniformly crystalline, giving the X-ray diffraction pattern of aniline picrolonate. Melting with decomposition occurred at 234° to 235°C. The preliminary data on depression of freezing point and the diffusion constant indicated that this compound has a low molecular weight (163). This requires further study. The biuret reaction of the secretin base was bright blue. Other color tests for protein (Millon, Hopkins-Cole, ninhydrin) were negative. Halogen, phosphorus and sulfur could not be detected. Elemental analyses of the picrolonate gave values consistent with the empirical formula C_3H_3ON. The material apparently had no free amino groups or reactive hydrogen because it failed to react with methyl magnesium iodide or acetyl chloride.

Greengard, Wolfrom and Ness (177) split their crystalline secretin picrolonate into soluble and insoluble fractions by extracting with warm nitroethane. Upon cooling of the soluble fraction, a crystalline material separated out which was devoid of secretin activity. This substance was subsequently identified as aniline picrolonate in the case of material not subjected to pyridine-ether recrystallization, and as pyridine picrolonate in the case of material to which such a recrystallization pro-

cedure had been applied. The nitroethane-insoluble fraction was an amorphous picrolonate which contained all of the secretin activity but which was not crystallizable except by retreatment with aniline or pyridine. These findings clearly show that the active crystalline picrolonates exist in the form of mixed crystals of either aniline- or pyridine-picrolonate with secretin picrolonate. Hammarsten and Ågren's crystalline picrolonate may be composed of similar mixed crystals because they too used pyridine as a solvent.

The fact that the potency of various batches of crystalline scretin picrolonate is relatively constant indicates that the material shows satisfactory constancy of composition. However, crystallinity is neither a necessary nor a sufficient criterion of high purity (208).

The secretin prepared as a crystalline picrolonate by Hammarsten and Ågren (194) appeared to be a polypeptide with a molecular weight of about 5,000 as determined in the ultracentrifuge. The amino acid composition of this material has recently been restudied by Edman and Ågren (98). By the use of paper chromatography the following amino acids were identified: lysine, arginine, histidine, crystine, glycine, serine, alanine, proline, valine, methionine, leucine, phenylalanine, tyrosine, aspartic acid and glutamic acid. Quantitative determinations of some of these amino acids were made and molar ratios were calculated. Neimann (345) applied Ågren's data to theoretical considerations of protein structure.

A curious property of the secretin prepared from the crystalline picrolonate of Ågren is that it can be partially digested by the enzyme aminopolypeptidase, with the opening of 10 peptide linkages, without loss of potency (3, 8). This calls to mind the similar property of thyroglobulin which can be digested with proteolytic enzymes to yield thyroxin which is still biologically active. The secretin of Hammarsten may bear the same relationship to that of Greengard as does thyroglobulin to thyroxin. Just as in the case of the thyroid, so in the case of secretin, we do not know which of these forms represents the circulating hormone, or indeed whether either of them does.

Greengard (165) has reviewed other procedures for the preparation of secretin (75, 327).

Secretin is dialyzable, as shown by the studies of Takács (428) on ultrafiltration and of Hammarsten and co-workers (194) on electrodialysis. The secretin picrolonates of Greengard and Ågren have about the same potency; the secretin base prepared from Greengard's picrolonate, however, is apparently about 4 times as potent as Ågren's (172). Krup (283) has prepared an amorphous secretin which appears to be several times more potent than Greengard's secretin base. These comparisons are based on interconversion of units and therefore cannot be considered to be exact. The preparation of crystalline secretin picrolonate by the method of Greengard has been confirmed by Doubilet (84) and by Babkin and Komarov (23).

Physiologic Properties. a) Pancreas. The most important action of secretin is the one which is best known, namely, stimulation of the flow of pancreatic juice. This it apparently accomplished by a direct action upon secretory cells, because the effect persists after denervation or transplantation of the gland. Gerard and Still (144), Kiyohara (260), Deutsch and Raper (78a), and Davies and co-workers (76a)

were able to detect a significant increase in the rate of oxygen uptake by isolated surviving slices of pancreatic tissue when secretin was added; however, Ågren (1), who used secretin prepared from his crystalline picrolonate, failed to confirm this finding.

Weaver (467) found that vasodilatin-free secretin stimulated pancreatic secretion without causing an increase in blood flow. Bennett and Still (34) showed that increased blood flow occurred only when there was some obstruction to outflow of the juice and a rise in the intraductal pressure. Duct distention may have been the cause of the parallel rise in blood flow which Gayet and Guillaumie (142) and Maltesos and Watson (321) found to accompany secretin activation of the pancreas. Artificial hyperpyrexia has been found to increase the pancreatic secretory response to secretin (349).

The relationship between the size of the dose of secretin and the quantity of juice secreted has been studied in dogs by Greengard, Stein and Ivy (173) and in man by Lagerlöf (289). The former group stated that an S-shaped curve was obtained when the volume response was plotted againt dosage, whereas Lagerlöf obtained a straight line for this relationship, probably because he explored only the middle region of the curve. The facts *1*) that the dose-response curve of secretin becomes a straight line over the entire range when the probit transformation (116) is applied, and *2*) that the ratio between the minimal effective dose and the dose producing maximal response is about 1 to 20 suggest that the dose-response curve may represent a distribution curve of threshold variations of individual elements responding in an all-or-none manner (64).

Greengard, Stein and Ivy (173) calculated that the minimum effective dose of secretin base prepared from the crystalline picrolonate injected at a constant rate was 0.0007 mg. per minute.

Estimation of the minimal amount of secretin required to stimulate the pancreas has also been made from the study of single rapid injections. Thus Ågren (1) calculated from the minimal effective dose of secretin which he found in cats (0.004 mg.) that the concentration of secretin in blood plasma could not be greater than 1 part in 40 million. With the highly purified secretin preparations studied by Krup (283) and found to be effective in dogs in doses as small as 0.005 mg., such a calculation gives a figure of 1 part in 100 million.

All who have investigated the problem are agreed that the pancreatic juice secreted in response to stimulation by secretin is low in enzyme concentration in contrast to the enzyme-rich juice produced by stimulation of the vagus nerve or administration of parasympathomimetic drugs (168, *inter alia*). However, the question of whether secretin causes any stimulation of enzyme secretion at all, or whether it stimulates only the secretion of water and inorganic materials has not been clearly answered until recently. The earlier studies in which preparations of secretin containing varying amounts of pancreozymin (see below) were used (29, 276, 295) must, of course, be discounted. Recently Wang and associates (463) used a highly purified preparation of secretin and found that once the enzymes which accumulated during the resting phase had been 'washed out' (22), then the output of enzymes per minute was unaffected by variations in the rate of flow of pancreatic juice caused by changing

the rate of secretin infusion. This strongly indicates that there is a basal rate of enzyme secretion which is unaffected by the rate of water secretion.

The concentration of bicarbonate in pancreatic juice has been shown (205, *inter alia*) to be a function of the rate of secretion and to be independent of the nature of the stimulus or of the enzyme content of the juice. In this respect the behavior of the bicarbonate of pancreatic juice is analogous to that of the hydrochloric acid of gastric juice.

It is well known that an abundant secretory response to secretin occurs after vagotomy, but no quantitative study of the effect of vagotomy on the response of the pancreas to secretin exists (309). The fact that vagotomy causes little change in the response to hydrochloric acid in the intestine (71) indicates that the action of secretin is affected but little.

Atropine causes little (436) or no (32, 99) depression of the response to secretin. Some results suggest that vagal nerve stimulation and parasympathomimetic drugs may act synergistically with secretin (143, 207, 434) but there is contradictory evidence (99, 320). It is probable that these contradictions arise from the fact that just as in the stomach (155) parasympathetic influences may be either augmentory or inhibitory depending upon the level of stimulation.

Ergotamine and dihydroergotamine markedly inhibit or abolish the response to injected secretin although they do not interfere with the response to epinine or prostigmine (167).

b) *Extra-pancreatic actions.* The only extra-pancreatic effect of secretin which is universally recognized is its ability to increase the rate of bile secretion by the liver. The earliest workers with secretin were aware of this effect and it has been abundantly confirmed (2, 286, 287, 328, 383), and only once denied (429). The stimulation of the liver and the pancreas by the same hormone calls to mind the embryological and phylogenetic relationships of these two glands. Ledrut and Ungar (296) showed that secretin stimulates the hepatopancreas of Octopus vulgaris. That secretin acts directly upon the liver was proven by Still and his co-workers (427) who showed that the effect persisted after removal of the pancreas and intestine. This was confirmed by Tanturi and co-workers (431). Although small doses of secretin are effective in stimulating bile flow, the maximal rate that can be attained with secretin stimulation is decidedly lower than with bile salts. The suggestions (135) that secretin is not the only biliary stimulant in intestinal extracts has not been confirmed. Ågren (1) gave small doses of secretin over long periods of time and interpreted his results as indicating that secretin caused an increase in the rate of secretion of bile salts and bile pigments. Others (328, 383) found that secretin stimulated the volume rate but not the output of solids. The results of studies in human subjects led Grossman and coworkers (186) to the conclusion that secretin is a hydrochloretic, like sodium dehydrocholate, stimulating the volume rate of bile flow without increasing the output of bile salts or bile pigments.

The suggestion that the biliary response during the secretin test with duodenal intubation be used as a clinical diagnostic aid in gall bladder disease (11, 79, 82, 91, 421) would appear to have some merit, but it has not been widely adopted.

The slight diuretic action of secretin (17, 288, 323, 350), which some have failed

to find (1), has been shown to be dependent upon the entrance of bile and pancreatic juice into the intestine (90).

All other actions which in the past have been attributed to secretin have been shown either not to exist or to be due to substances other than secretin present in the cruder extracts. Thus the purified secretin of Ågren (1) was shown to be free of gastric stimulants, hypoglycemia-producing substances, vasodilatins, cholecystokinin and smooth muscle stimulants.

Metabolism of Secretin. The effectiveness of secretin by intramuscular, subcutaneous and intraperitoneal administration is very low, even when many times the intravenously effective dose is given (1). Slight absorption occurs when it is instilled into the rectum (86). Secretin is inactivated by gastric and pancreatic juices and is totally ineffective when given by mouth (216). This has usually been attributed to digestion by proteolytic enzymes but in the only experiment which this reviewer has found recorded in the literature in which secretin was incubated with crystalline pepsin or trypsin no inactivation occurred (174).

Greengard and his co-workers (174) studied the inactivation of secretin which occurred when it was incubated with blood serum or plasma. They found that inactivation increased with time and occurred between *p*H 5 and 8, and that the optimal temperature was in the neighborhood of body temperature. Heating the serum to 60°C. destroyed its ability to inactivate secretin. On the basis of these findings they suggested that the inactivation was enzymatic and that the principle be designated secretinase. A similar secretin-inactivating principle was demonstrated to be present in urine (175) and it probaly is present in digestive secretions, accounting for their ability to destroy secretin. Doubilet (84) reported that vitamin K inhibited the action of secretinase both *in vivo* and *in vitro*. Although these results suggest the presence of a specific enzyme, they are not conclusive in this respect.

Clinical Use. Since 1926 (63) secretin has been used in human subjects as a stimulant for pancreatic function tests (12, 61, 81, 79, 80, 290, 294) and for physiological studies (9, 10, 68, 69, 193, 255, 293, 458, 62, 136). Although every clinic which has used the test has reported that it is of value in detecting disease of the pancreas and that the response to secretin may in some cases be abnormal when all other tests or signs of pancreatic function are normal, the test is still not in general use. This is probably due to the unavailability, until recently, of a suitable secretin preparation. Now that the several commercial preparations of secretin suitable for administration to human subjects are available, it is probable that the test will gain the popularity it has been shown to deserve.

In infants, the test is of crucial value in differentiating celiac disease from chronic cystic fibrosis of the pancreas (317,355). In adults it is useful in detecting loss of functional pancreatic tissue which may occur as a result of carcinoma or chronic relapsing pancreatitis. In the early stages of pancreatic disease the test may not reveal an alteration from normal. When the test begins to show deviations from normal a diminution in the volume of pancreatic juice and in its concentration of bicarbonate are first seen. In the later stages, diminution of enzyme concentration also occurs (83). If the pancreatic ducts are partially or completely obstructed, injection of secretin leads to a rise in serum amylase, provided the pancreas has not undergone

complete atrophy (363). A clinical test based on the production of functional obstruction to the ducts, presumed to result from the spasmogenic action of morphine administered before injection of secretin, has been devised by Lagerlöf (291, 292). Myhre and co-workers (335) showed that Mecholyl could be used to replace morphine in this test. The Mecholyl-secretin or morphine-secretin test produced a rise in serum enzymes in 70 per cent of normal subjects and failed to produce such a rise in any of nine subjects with far advanced pancreatic disease who presumably had atrophic pancreases.

PANCREOZYMIN

Definition. Pancreozymin (Harper and Raper, 1943, 202) is the hormone which stimulates the secretion of enzymes by the pancreas. It is released by the upper intestinal mucosa in response to the presence of certain chemical substances.

Proof of Existence. It has long been recognized that the pancreatic juice secreted in response to some foods is rich in enzymes whereas other agents, such as HCl, stimulate the secretion of a watery juice low in enzyme content. Until recently, the most widely accepted theory of the means of regulation of the enzyme content of the pancreatic juice was that of Mellanby (326) who proposed that secretin stimulated the flow of pancreatic juice very low in enzymes and that the concentration of enzymes in the juice was regulated by the vagus nerves. That vagal stimulation leads to augmented enzyme secretion by the pancreas is well established (72, 303, 354). When, however, Harper and Vass (203), who worked with anesthetized cats, and Crider and Thomas (71), who studied unanesthetized dogs, both showed that certain foods were still capable of producing stimulation of secretion of pancreatic enzymes after bilateral vagotomy and sympathectomy, the existence of a mechanism of control other than the extrinsic nerves was demonstrated. Harper postulated that this factor controlling enzyme secretion was a hormone and he proceeded to demonstrate the presence in intestinal extracts of a substance capable of augmenting enzyme secretion by the pancreas. Thomas (434) favors the view that local nervous connections between the duodenum and the pancreas, the existence of which was originally postulated by Popielski (362), are responsible. To prove that such nervous connections between the intestine and pancreas are not necessary for the stimulation of enzyme secretion, experiments must be performed using the subcutaneously autotransplanted pancreas. Farrell and Ivy (107) performed a few such experiments with equivocal results but the methods for measuring enzyme concentrations which they used were inadequate and this work should be repeated. Preliminary studies by Wang and Grossman (462) show that substances capable of evoking enzyme-rich pancreatic juice, such as peptone, continue to show this action in dogs with the pancreas transplanted under the skin.

Until more complete evidence of this kind is available the existence of pancreozymin cannot be considered to have been rigorously proven, although the evidence already available is quite convincing.

Mechanism of Release. a) Site of formation. Harper and Vass (203) found that foods must leave the stomach and enter the intestine in order to stimulate pancreatic

enzyme secretion. Harper and Raper (202) were able to extract pancreozymin from the upper intestinal mucosa but found none in the gastric or lower ileal mucosa.

b) Stimuli for release. Soap, peptone (71, 440), starch, dextrin, maltose, lactose (442), casein, inulin, saline and distilled water (203) have been stated to be capable of increasing the enzyme output of the pancreas. Presumably all of these substances are capable of causing release of pancreozymin, but studies on pancreatic transplants are needed to prove this assumption.

c) Effect of atropine. Atropine depresses the concentration of enzymes in the pancreatic juice regardless of the stimulus which is acting, whether it be secretin, hydrochloric acid in the duodenum, or foodstuffs (441). Atropine does not interfere with the action of injected pancreozymin (201). As in the case of gastrin, these facts suggest that atropine is capable of preventing the release of pancreozymin. Further evidence on this point is needed.

Preparation. Pancreozymin has been prepared by several procedures from the by-products arising from the purification of secretin from intestinal extracts. Harper and Raper (202) extracted intestinal mucosa with absolute alcohol, removed the alcohol by vacuum distillation, and precipitated the secretin by the addition of bile salts and acetic acid. The filtrate from this treatment was saturated with sodium chloride and the resulting precipitate extracted with absolute alcohol; the material obtained by evaporating this extract to dryness contained the pancreozymin activity. Harper and Raper pointed out that the SI preparation of Greengard and Ivy (170) contained pancreozymin as well as secretin. The latter authors (169) found that the precipitate obtained by addition of aniline to SI in 80 per cent acetone solution contained all of the pancreozymin whereas most of the secretin was in the supernatant fluid. From this precipitate, traces of secretin, as well as other impurities, could be removed by extraction with acidified methyl alcohol (171). Both Greengard and Ivy (171) and Harper and Raper (202) obtained preparations which were effective in doses as low as a few milligrams.

Harper and Raper (202) observed no loss of activity on incubation of their product with U.S.P. pepsin, but reported complete destruction with activated pancreatic juice.

Physiologic Properties. Purified preparations of pancreozymin freed of secretin have no effect upon the volume rate of pancreatic secretion, producing only an increase in enzyme content of the juice. Harper and Mackay (201) showed that pancreozymin could produce depletion of the enzyme granules in the acinar cells comparable to that caused by vagal stimulation, and that repeated injections of pancreozymin were able to sustain the enzyme concentration at high levels as long as they were continued, up to 4 hours in some experiments. Pancreozymin augments the output of all of the pancreatic enzymes (tryptase, amylase and lipase). This parallelism of the variations in the pancreatic enzyme secretion also occurs with other stimuli such as vagal nerve stimulation or administration of parasympathomimetic drugs (31, 168, 169, 203). Wang and co-workers (463) found that the content of alkaline phosphatase in the pancreatic juice was uninfluenced by pancreozymin and histological evidence suggested that this enzyme is secreted by the centroacinar

and small ductule cells of the pancreas. This and other evidence has led to the suggestion (184) that the centroacinar and ductule cells as well as the acinar cells are secretory elements, and that secretin acts upon the former and pancreozymin upon the latter. Further evidence is required before this theory can be considered to be proven.

Davies and co-workers (76a) showed that pancreozymin, like secretin, increases the rate of oxygen uptake of surviving pancreatic slices.

Purified pancreozymin preparations are free of secretin, cholecystokinin, vasodepressor and hypoglycemic activities. Inactivation of pancreozymin by blood serum has been demonstrated (169).

Assay. Harper and Raper (202), using anesthetized cats with cannulated pancreatic ducts, injected pancreozymin-free secretin every ten minutes and then gave periodic injections of pancreozymin. The enzyme content of the pancreatic juice rose as much as five-fold and usually returned to the basal levels within 30 minutes. Greengard (171) proposed a complicated quantitative assay method involving fractionation of the response to single secretin injections. Burn and Holton (54) showed that when periodic pancreozymin injections were superimposed upon continuous secretin stimulation the output of enzymes per unit time (expressed as volume times logarithm of the diastatic power) was rectilinearly proportional to the dose of pancreozymin. This is a promising method of assay.

Use in Man. Pancreozymin has not yet been administered to human beings. It may be found to have some value as a testing agent to be used with secretin.

CHOLECYSTOKININ

Definition. Cholecystokinin (Ivy and Oldberg, 1928, 237) is the hormone which is released by the upper intestinal mucosa when certain chemical substances are in contact with it and which causes the gallbladder to contract and empty.

Proof of Existence. Our present knowledge of the physiology of the gallbladder has been hard won. A surprisingly few years ago the contention was being voiced that the gallbladder did not empty via the cystic duct at all (217) and finally, when this point was ceded, as recently as 1932 strong doubt was expressed as to whether the contraction of the muscle of the gallbladder wall assisted in the emptying of the viscus (192, 324). Today, however, it is generally recognized that contraction of the muscular wall of the gallbladder causes the contents to empty via the cystic duct, and that cholecystokinin is the principal regulator of this contractile activity.

It has been known for many years that emptying of the gallbladder occurs when certain foods or dilute acid are present in the upper intestine. The concept of a hormonal mechanism for gallbladder contraction became established with the demonstrations *1*) that the extrinsically denervated gallbladder evacuates normally in response to food, *2*) that in cross-circulation experiments, the gallbladder of a recipient dog contracts when acid is placed in the duodenum of the donor and *3*) that vasodilatin-free extracts of intestinal mucosa can cause contraction of the gallbladder (216, 217). Important confirmatory evidence was added by *1*) the autotransplantation experiments of Houssay and Rubio (211) in which the gallbladder grafted into the neck by vascular anastomosis was found to respond to acid in the duodenum,

and *2*) the studies of Sandblom (387) in which he observed radiographically the emptying of the gallbladder in recipients of blood transfusions from donors who had been fed fat. The possibility that the absorbed products of digestion of fat might act directly as stimulants of gallbladder contraction has been clearly ruled out by extensive experiments (459, 460).

Taken together these pieces of evidence constitute imposing proof for the existence of cholecystokinin (hereafter designated CCK), the name proposed by Ivy and Oldberg (237) for the hormone producing gallbladder contraction.

Mechanism of Release. a) Site of formation. The most effective site for the action of substances which cause the gallbladder to contract is the upper part of the small intestine and the mucosa of this region is the richest source of CCK (217). Occasionally the gallbladder can be incited to contract by stimuli confined to the stomach (332). Small amounts of CCK have been extracted from the gastric mucosa (93). Egg yolk introduced into the ileum or rectum of human subjects failed to cause the gallbladder to empty (49) and CCK could not be found in colonic mucosa (217). The duodenal mucosa of the horse, an animal without a gallbladder, apparently does not contain CCK (217). It has been found to be present in the upper intestinal mucosa of the hog, dog, sheep, cattle (233), rabbit and man (93).

b) Stimuli for release. Ivy (217) presented a long list of substances capable of causing the gallbladder to contract when present in the upper intestine. The strongest stimulants are fat and fatty acids, dilute hydrochloric acid and peptone. A humoral mechanism has not been proven for each of these, but there is no reason to believe that they act in any way other than by causing cholecystokinin to be released. Atropine has no important effect upon gallbladder emptying in response to foods nor does it alter the effect of injected CCK (236, 315); it may be assumed, therefore, that neither the release of CCK nor its action on the gallbladder involves cholinergic mechanisms.

Preparation and Chemical Properties. CCK shows a strong tendency to follow secretin in fractionation procedures. Preparations of secretin free of CCK, and preparations of CCK free of secretin have, however, been made in several laboratories (4, 233). A method of preparation permitting complete separation of the two hormones with good recovery of both has not been accomplished, so that preparation of CCK-free secretin entails the loss of much of the CCK and vice versa. Ågren (4) has taken advantage of the difference in isoelectric point between secretin and CCK (7.75 and 5.0 to 5.5, respectively) to effect separation electrophoretically. His purest product had no detectable secretin activity and did not depress the blood pressure; in doses of 2 mg. it caused an isolated guinea pig's gallbladder to contract. Ivy *et al.* (233) extracted a preparation containing both CCK and secretin with absolute alcohol in a Sohxlet apparatus and found that the residue contained only CCK. Greengard and Ivy (170) and Doubilet (84) reported that the n-butyl alcohol extract of purified CCK-secretin preparations had CCK but no secretin activity. Ivy *et al.* (233) found that their preparations did not dialyze through celloidin membranes but Ågren (4) observed dialysis through cellophane.

Assay. Two general types of assay procedures have been used. In the first, the intralumenal pressure of the obstructed *in situ* gallbladder of the dog is recorded

(236), and in the second the contraction of the isolated gallbladder of the guinea pig is observed in a smooth muscle chamber (4). The first method is more sensitive and more specific. Gershbein and co-workers (146) have standardized the procedure, using modern principles of bioassay. The older unit of Ivy and co-workers (226) was defined as the amount of vasodilatin-free extract which will cause on intravenous injection a more or less immediate one cm. rise in intra-gallbladder pressure. Gershbein *et al.* (146) defined the unit as the activity contained in 1 mg. of a standard reference powder (SI, a preparation containing both secretin and CCK and used as a standard for both). The ratio between the threshold doses by the *in vivo* and *in vitro* methods is highly variable. For example, Jung and Greengard (242) found it to be 1 to 50, whereas Doubilet and Ivy (85) found that under their experimental conditions it was 1 to 0.25. It is obvious that assumptions such as Ågren's (4), concerning *in vivo* potency based on results of *in vitro* assay, are likely to be inaccurate.

Seager (408, 409) has demonstrated that the gallbladder of the frog shows a contractile response to the intracardiac injection of preparations of CCK and has proposed the use of this as an assay procedure. By inserting a fine glass cannula directly into the common bile duct of unanesthetized dogs via a large cannula in the duodenum opposite the papilla, Snape, Friedman and Thomas (422) made direct recordings of biliary tract pressure and used this as an assay method for CCK. Ågren (4), Seager (408) and Snape (422) have defined units for their methods of assay.

Physiologic Actions. Within about 1 minute after the intravenous injection into a dog of an appropriate dose of an active CCK preparation (e.g., 2 mg. of a purified preparation such as that used by Lueth *et al.* (315), the intralumenal pressure begins to rise, reaching a peak of 10 to 20 cm. of bile pressure within another 2 to 3 minutes, and then gradually returns to the original basal level over a period of 10 to 30 minutes. A sub-threshold dose of CCK may induce rhythmic tonus changes without causing a sustained contraction. Injection of a small dose of CCK every ten minutes for one hour will cause complete evacuation of the gallbladder of an unanesthetized dog (236). Gershbein and co-workers showed that the shape of the curve relating the dose of CCK to intralumenal pressure rise is essentialy a straight line until a maximal response is attained. There is an optimal intralumenal pressure for maximal force of contraction such that if the pressure within the gallbladder goes above or below this value, the contractile response to CCK is diminished (85).

Snape (420) showed that vagotomy increased the length of the latent period for gallbladder contraction when fat in the duodenum was the stimulus, but not when CCK was injected intravenously. This suggests that activity of the vagus may facilitate the formation and release of CCK by the duodenal mucosa, although, as already pointed out, the lack of effect of atropine does not support this possibility.

CCK has been observed to cause contraction of the gallbladder in the dog, cat (236), frog (409), rabbit (461) and man (315).

Since injection of CCK causes gallbladder evacuation, it is a reasonable assumption that relaxation of the sphincter of Oddi occurs simultaneously. Direct studies on the effect of CCK on the activity of the sphincter of Oddi are complicated by the stimulation of duodenal motility which is caused by preparations of CCK. For this reason the evidence must be considered inconclusive, but there is some indication

that when the activity of the sphincter is not masked by duodenal motor activity, CCK causes the sphincter to relax (388). This problem could probably be profitably approached by studying the effect of CCK on the isolated sphincter muscle by the method of Magee (318).

The presence in CCK preparations of this constituent which stimulates intestinal motility, referred to above, raises several questions. Is this activity due to CCK or to a separate constituent of the extract? The answer to this question waits upon further purification of CCK. It is well known that intestinal extracts, and particularly CCK preparations, contain a substance, or substances, which cause isolated smooth muscles such as that of the intestine (242) and spleen (113) to contract. The suggestion of Bjurstedt and co-workers (37) that the activity of CCK preparations is due to *substance P*, a general smooth muscle stimulant extractable from a variety of tissues, is not supported by the facts *1*) that CCK has been extracted only from the gastrointestinal mucosa and *2*) when purified, CCK exerts no vasodepressor action whereas ability to produce fall in blood pressure is one of the identifying properties of *substance P*. The most important question in regard to this motor excitant is: Has it a physiological role or is it merely an artifact created by the extraction procedure? The question of the existence of a hormone for intestinal motor activity will be considered in a separate section of this review.

Although gallbladder evacuation has been produced by the intravenous injection of a CCK preparation in human subjects (226), a preparation pure enough for routine clinical use is not available. It is not unlikely that such a preparation would be of distinct value for clinical use in testing for motor disorders of the biliary tract and in establishing the diagnosis of biliary dyskinesia (224).

Greengard, Stein and Ivy (176) demonstrated that blood serum inactivates CCK, just as it inactivates secretin, and that the mechanism of inactivation is probably enzymic in both instances.

The impairment of gallbladder emptying which is known to occur during pregnancy has been studied in the guinea pig, and it has been shown that in animals in the second half of pregnancy the degree of emptying of the gallbladder in response to a standard dose of CCK is distinctly less than in normal control animals (329, 419). The suggestion has been offered that an increased rate of destruction of CCK by the blood serum may be involved (419).

ENTEROGASTRONE

Definition. Enterogastrone (Kasaka and Lim, 1930, 277) is the hormone which is released by the mucosa of the upper small intestine when an adequate concentration of fat or sugar is in contact with the mucosa, and which inhibits the motor and acid secretory activity of the stomach.

Proof of Existence. The original observation leading to the discovery of enterogastrone was made by Ewald and Boas (104). While studying the effect of various test meals on gastric function in human subjects, these investigators noted that the addition of olive oil to a gruel test meal caused, in comparison with gruel alone, depression of the acid concentration of the gastric contents and delay in the emptying of the meal from the stomach. Several years later these facts were essentially confirmed in

studies on dogs by workers in Pavlov's laboratory (250), and the additional and significant observation was made that fat acted only in the duodenum to produce depression of gastric activity, and that when it was confined to the stomach no such depression occurred. Documentation of the early history of this subject can be found in the papers by Ivy and Gray (229) and Sandweiss (391).

The first insight into the mechanism of this inhibitory action of fat was gained from the studies of Farrell and Ivy (106) who showed that the feeding of olive oil to a dog with a subcutaneously autotransplanted pouch of the fundic portion of the stomach caused inhibition of the motor activity of the pouch. Shortly thereafter Feng, Hou and Lim (110) demonstrated that acid secretion was also inhibited under similar circumstances. It was thus established that nervous connections between the duodenum and the stomach were not necessary for this effect; this indicated that it was humoral in nature. With the demonstration that neither gastric secretion (110) nor gastric motility (373) was inhibited by intravenous injection of fatty chyle, it became apparent that the humoral agent was not an absorbed digestion product of fat, and the suggestion that it was hormonal was supported. The name 'enterogastrone,' derived from entero/n, gastr/on, chal/one, was proposed by Kosaka and Lim (277). When certain extracts of the upper intestinal mucosa were shown to be effective upon parenteral administration in mimicking the gastric inhibitory action of fat in the intestine (278), the final step in the proof of the existence of enterogastrone was taken. It will become evident in what follows that this last, the action of the intestinal extracts, is the weakest link in the chain of evidence establishing the existence of enterogastrone. This is so because, despite the large amount of work on the subject, certain questions concerning the specificity of these extracts—to be considered below—have not yet been satisfactorily answered. Nevertheless, the purely physiological evidence on the endogenous release of enterogastrone is so convincing that the existence of this hormone may be considered to be firmly established.

Mechanism of Release. a) Site of formation. As already stated, fat confined to the stomach does not inhibit gastric activity, but does when introduced into the duodenum (147) or jejunum (466). No direct studies have been made on the relative effectiveness of fat confined to various levels of the intestine. Quigley and Hallaran (368) showed that glucose solutions inhibited gastric motility when placed in the duodenum but did not do so when confined to the lower ileum, the colon, or the stomach, and, therefore, the assumption is probably warranted that, like secretin and CCK, enterogastrone concentration decreases caudalward along the intestine.

Kosaka *et al.* (280) compared the effectiveness of extracts from various tissues in inhibiting gastric secretion. Extracts from intestinal and colonic mucosa were about equally effective, whereas those from smooth muscle, striated muscle, and gastric mucosa were less so, while those from blood and liver were inactive. Of course, the interpretation of these results must be made with reservation because the possibility that non-specific inhibitors were present cannot be ruled out.

b) Stimuli for release. Motor. Although fat was the first substance that was recognized as being capable of releasing enterogastrone, it is probable that a number of other substances can do this. Quigley and Meschan (370) showed that soaps are more effective than fatty acids, which in turn are more effective than neutral fats

in causing motor inhibition of the pyloric part of the stomach. Because these studies were performed on intact dogs, the possibility that some or all of the effect was due to nervous reflexes (the enterogastric reflex of Thomas, 443) must be considered. All investigators who have studied the question agree that mineral oil is ineffective in producing gastric motor inhibition (57, 94, 370). Some have claimed that short-chain fatty acids are more effective than long-chain ones (475), but others have been unable to confirm this (57, 370). Similarly, the statement has been made that unsaturated fatty acids are more effective than saturated ones (380, 444), but the careful studies of Card (57) and of Quigley and Meschan (370) did not bear this out. All who studied the point recognized that oleic acid was more effective than other fatty acids (57, 370, 380, 444); the generalization that unsaturated fatty acids are more potent is, however, apparently not valid.

The effect of impairment of pancreatic digestion on the release of enterogastrone by fat requires further study (346).

The only substances other than fat which have been shown to be capable of inhibiting the transplanted stomach pouch are the sugars, glucose, sucrose and lactose (371). These sugars exerted a significant effect only when they were present in hypertonic concentrations (148, 241, 376). Other hypertonic solutions, such as hypertonic solution of sodium chloride (148), inhibit gastric activity, but it has not been determined by studies with transplanted pouches whether this is due to release of enterogastrone. Thomas and his co-workers (437, 443) have shown that acid and the products of protein digestion also act in the duodenum to produce gastric motor inhibition, but these substances apparently act through a nervous mechanism, the enterogastric reflex, which does not occur in the absence of the vagus nerve. The possibility that fats and sugars act in part by the enterogastric reflex has been considered by Quigley and co-workers (371, 373) who found that inhibition of motor activity began slightly sooner, and was somewhat more pronounced, in the intact stomach than in the vagally denervated stomach. It is unknown whether this was due to partial dependence on the enterogastric reflex or to reduced susceptibility of the denervated stomach to the humoral agent. In this connection, the findings of Antia and Ivy (19) are significant. They found in dogs that the delay in gastric emptying caused by addition of oil to a liquid test meal was just as great after vagotomy as in the intact animal.

Secretory. Among the agents which inhibit gastric secretion when present in the duodenum, fat is the only one which has been proven by experiments with the transplanted pouch to act by release of enterogastrone. Concentrated sugar solutions which have been shown to be capable of inhibiting gastric secretion when present in the duodenum (77, 414), probably also act by releasing enterogastrone just as in the case of motor inhibition by sugars. Curiously, although gastric motor inhibition by acid in the duodenum depends on the integrity of the vagi, the gastric secretory inhibition apparently does not (356). This phenomenon deserves further study.

Assay. a) Motor inhibition. Balloons in the stomach of intact dogs or of dogs with simple gastric fistulas have been used in assaying the motor inhibitory action of intestinal extracts. Since it has recently been demonstrated, however, that the preparations so far tried do not inhibit motility in the vagally denervated stomach

(204), it is apparent that they do not reproduce the action of fat in the duodenum and therefore probably do not contain the physiologic hormonal agent. Hence, all of the extensive and painstaking work which has been done on inhibition of gastric motility by intestinal extracts must be set aside and not applied to the problem of the nature of the inhibitory agent, since it is probable that this action is due to a non-specific substance and not to enterogastrone. Obviously the assay procedure cannot be perfected until true hormone-like extracts are available.

b) *Secretory inhibition.* Several fundamental difficulties are encountered in assaying gastric secretory depressants. In the first place, it is necessary to deal simultaneously with variations in the effectiveness of both the stimulatory agent and the inhibitory agent. In the second place, several non-specific factors may contribute to the inhibition. For example, such events as nausea and vomiting, hypotension or pyrexia, which may occur as side effects in response to the injection of certain partially purified extracts, are capable of markedly inhibiting gastric acid secretion even in the transplanted gastric pouch (190). It has been shown that pyrogens in subpyrogenic doses can inhibit gastric motility (343) and it has been claimed (341, 342) that subpyrogenic doses also inhibit gastric secretion, and that much of the inhibition caused by intestinal extracts may be due to their content of pyrogen. On the other hand, however, *1*) preparations of intestinal extracts free of pyrogen, even when tested in multiples of the secretory depressant dose, have been prepared and *2*) it has recently been shown (38) that subpyrogenic doses of pure bacterial pyrogens do not inhibit gastric secretion. Nevertheless, it cannot be emphasized too strongly that because of the frequency with which contamination with pyrogen occurs, the body temperature of the animals should be recorded repeatedly during every assay on enterogastrone preparations. Very few of the papers on enterogastrone give information on the pyrogenic activity of the extracts used. Because of this fact, and because pyrogen is a common contaminant of enterogastrone preparations, it is not possible to know how much of the observed secretory depression was due to pyrogen. This reservation must be applied to almost all the work with enterogastrone extracts discussed in this review.

The two general types of method which have been used to assay gastric secretory depressants are the 'two-dose histamine method' and the 'continuous histamine method.' For quantitative results the latter is unquestionably superior because *1*) the day-to-day and hour-to-hour variation in the secretory response of the dogs is less and *2*) the time of onset and duration of action of the inhibitor can be determined. The method essentially is as follows: a small dose of histamine is administered subcutaneously every ten minutes during the entire test to dogs with Heidenhain pouches or total gastric pouches; after the secretory response has become stabilized (one to two hours after beginning), secretion is collected for a control hour; then the inhibitory agent is injected and the depression of secretion is noted. Code and co-workers (66) have recently given a detailed description of their modification of Gray's continuous histamine assay method. They recommended that every ten minutes a dose of histamine large enough to produce a certain fixed percentage of the maximal secretory capacity be given. Studies by Grossman and co-workers (180) indicated that it is preferable to fix the dose of histamine rather than the secretory response, but

further evidence on this point is needed. Since, however, it can be shown that by the use of either of these modifications of the continuous histamine method, the graphs relating logarithm of dose to percentage inhibition are linear, both methods of measurement are apparently reliable.

Gray, Bradley and Ivy (151) defined the unit of enterogastrone as that quantity which, when injected intravenously in a 12 to 14 kg. dog with a pouch of the entire stomach receiving sufficient histamine subcutaneously at 10-minute intervals to maintain a uniform flow of 1 cc. of gastric juice (5 mg. HCl) per minute, causes a 50 per cent reduction in secretion of hydrochloric acid during two hours following the injection. The question of the validity of using a fixed rate of secretion has already been considered.

Uvnäs (451) was unable to depress gastric secretion in cats by injection of an enterogastrone preparation. Probably he used an inactive material, since Howat and Schofield (212) who devised a carefully standardized two-dose histamine test for use in anesthetized cats, showed that enterogastrone preparations did produce inhibition by this method.

The measurement of gastric secretion in rats with the pylorus ligated (133) has been used as an assay method for enterogastrone (245, 456). The results of assay of a series of preparations by both the 'continuous histamine' method in dogs and the pyloric ligation method in rats gave rather poor correlation (456).

Preparation and Chemical Properties. The methods which have been used to prepare enterogastrone concentrates resemble those used in the preparation of secretin and cholecystokinin. The method of Gray, Bradley and Ivy (151) is most widely used. It consists essentially of isoelectric precipitation of an aqueous suspension of the A precipitate (see section on SECRETIN), precipitation of the activity by addition of tannic acid, extraction of the precipitate with acidified aqueous acetone and precipitation with an excess of acetone. Further purification with picric acid has been described by Greengard *et al.* (166). Material prepared by this method produces an average of 50 per cent inhibition of acid secretion when injected intravenously in 25 mg. doses in pouch dogs receiving 0.02 mg. of histamine diphosphate every ten minutes.

All enterogastrone concentrates so far prepared contain amino acids, although the molecular size of the aggregates in the purified preparations is too small to allow precipitation with trichloracetic acid. Visscher (455) and Winberg (477) showed that the active principle is dialyzable through cellophane membranes. Purification by electrophoresis has been attempted (347, 455) and the results have indicated that the least mobile fraction is the most active.

Kosaka's enterogastrone extracts contained no secretin. Vasodepressor activity is often present (151, 302) but preparations entirely devoid of it have been made (302). Substances stimulating gastric secretion occur as contaminants in enterogastrone preparations (151, 451, 477). Several methods of removing them have been proposed (145, 477).

Physiologic Properties. a) Endogenous enterogastrone. In order for fat or sugar to produce inhibition of gastric activity it must reach the duodenum in a concentration sufficiently high to cause enterogastrone release. In human subjects, Annegers and

Ivy (18) found that in all but 3 of 30 subjects increasing the fat of a mixed meal from 6 per cent to 14 per cent (calculated on a wet weight basis) caused a significant decrease in rate of gastric evacuation. Van Liere and associates (453) showed that the addition of as little as 25 gm. of glucose to a 200 cc. test meal containing farina and barium sulfate significantly delayed emptying in human subjects and that, as the quantity of glucose added was raised, the delay increased. A similar test meal was used by Henschel and co-workers (206) but they replaced part of the oatmeal isocalorically with fat or sugar. No delay in emptying was seen even when fat accounted for 70 per cent of the calories of the test meal; in this diet, however, fat represented only 4 per cent of the wet weight. Similarly, in the sucrose-substituted diet, sucrose represented only 5 per cent of the wet weight. It is probable that the threshold concentration for release of enterogastrone can best be expressed in terms of weight of fat or sugar per unit volume. The high threshold for gastric inhibition by sucrose was confirmed by the studies of Hunt and Spurrell (215) who found that it was necessary to add 100 gm. of sucrose to a one pint test meal in order to cause a significant delay in emptying. When this result is compared with those of Van Liere *et al.* (453) who used glucose, it is apparent that glucose is more effective than sucrose in delaying gastric emptying. Whether this difference is due solely to osmotic differences is not known.

The latent period between the introduction of fat into the intestine and the onset of motor inhibition varies with the concentration and chemical nature of the fat or sugar. It may be as short as one minute or as long as six or seven minutes (373). The duration of inhibition of motility varies with the amount of inhibitory agent; Card (57) found a linear relation over a rather wide range between dose of oil introduced into the duodenum and duration of absence of contractions. Quigley and coworkers (373) found that in the dog one egg yolk produced quiescence of the main stomach or of an autotransplanted stomach for from 30 to 40 minutes; in the human, Card (57) showed that 2 cc. of linseed oil introduced directly into the duodenum caused gastric motility to stop for 20 minutes.

When the inhibiing agent is strong enough the basal tone falls during the period of cessation of contractions. All types of gastric motility, including hunger contractions and contractions in response to distention or to a parasympathomimetic drug, are inhibited by substances which release enterogastrone, but the stronger the initial stimulus for contraction the smaller is the degree of inhibition (371).

The older idea that fat delays gastric emptying by causing the pyloric sphincter to close has now been discarded in view of the clear demonstration that the mechanism is one of inhibition of contractions of the entire stomach including the pyloric sphincter (370, 372, 436). Discharge of gastric contents is suspended because of decrease in propulsive force.

Accurate information on secretory inhibition by endogenous enterogastrone, in regard to the latent period for onset of action and duration of action, is somewhat more difficult to obtain than in the case of motor inhibition. In general it is necessary to use much larger doses of inhibitory agent to detect secretory inhibition. This is probably due to the fact that strong stimulants are used to induce secretion and, therefore, a strong inhibitory agent is needed. Furthermore, in contrast to motor in-

hibition, short periods of gastric secretory inhibition, lasting, for example, two or three minutes, cannot be accurately detected, so that doses of fat or sugar which produce much longer lasting effects must be used. To give some idea of the duration of inhibition of secretion produced by fat, the data of Gray, Bradley and Ivy (151) may be used. These workers found that 50 cc. of olive oil depressed the acid secretory response to a meat meal in Pavlov pouch dogs by more than 50 per cent for three hours. Fat in the duodenum is capable of inhibiting all types of gastric secretion including interdigestive basal secretion, and the secretory response to sham feeding, insulin, parasympathomimetic drugs, food and histamine (299).

The addition of one ounce of oil to an Ewald type test meal usually (26, 70, 304, 379) but not invariably (20, 55) moderately reduces the gastric secretory response. Killian and Knapp (251) used large quantities of fat in their test meals but, in contrast to all previous investigators, observed no secretory inhibition. As with motor inhibition, it is clear that the degree of secretory inhibition decreases as the strength of stimulus increases; thus, for example, a given dose of fat caused more inhibition of the secretory response to a meal than of that to histamine (151).

Since the concentration of acid in gastric juice is related to the rate of secretion, it would be anticipated that, as the volume of juice falls due to the action of endogenously released enterogastrone, the concentration of acid would also fall. This is, in fact, what occurred in the experiments of Gray, Bradley and Ivy (151). The output of neutral chloride and of mucin (measured as reducing substances after hydrolysis) was, however, unchanged.

Fat also causes a marked fall in the concentration of pepsin in the gastric juice (15, 16, 303, 413). This inhibition of pepsin secretion is apparently not due to the release of enterogastrone, but is a nervous phenomenon, because it failed to occur after vagotomy (183).

b) *Exogenous enterogastrone.* As already mentioned, the enterogastrone extracts that have thus far been prepared inhibit gastric motility only in the vagally innervated stomach (151, 279) and, therefore, cannot be considered to contain the true hormonal substance. There is nothing to suggest a physiologic function for this inhibitor. The motor inhibitions produced by D-amphetamine (401) and by the enterogastric reflex (443) also require the presence of the vagus. The physiologic evidence suggests that a substance capable of inhibiting motility in the denervated stomach should be extractable from the intestinal mucosa, and search should be made for this substance.

The intravenous injection of about 25 mg. of a potent enterogastrone preparation will cause about 50 per cent depression in the output of acid secretion during a 'continuous histamine' test. Inhibition begins within 10 minutes after injection and lasts for about three hours (229). Öbrink (348) found that injection of enterogastrone depressed neutral red excretion to the same extent as acid secretion. Side effects such as fall in blood pressure, collapse, vomiting or pyrexia are due to impurities and do not occur with the most purified preparations. The secretory depression is specific for the stomach. No effect on salivary, pancreatic or biliary secretion occurs (151). As with the other gastrointestinal hormones, intravenous administration is much more effective than subcutaneous or intramuscular; the oral route is inef-

fective in dogs (151) and humans (360). Some preparations contain a stimulatory substance and they may actually increase acid secretion when given in large doses subcutaneously or intramuscularly. Since only the intramuscular route has been used in human subjects, large doses of material purified so as to remove stimulatory substances must be used. Most investigators who have studied the effect of enterogastrone on human gastric secretion have observed little or no depression of secretion (111, 257, 297, 360). Using a dialyzed preparation (and thus presumably relatively free of stimulatory factors) Kirsner *et al.* (259) found that it was necessary to give 5 gm. intramuscularly to obtain consistent and significant depression of nocturnal basal acid secretion in human ulcer patients. These same investigators (258) showed that non-specific protein (from milk) did not produce significant depression. When a highly purified preparation of enterogastrone, capable of inhibiting gastric secretion in humans in small doses, becomes available, it is likely to be useful in the management of acute exacerbations of peptic ulcer. The use of intestinal extracts in the treatment of peptic ulcer is considered in the discussion of anthelone.

Davenport and co-workers (76) found that when an enterogastrone preparation was added to the salt solution used to bathe the isolated stomach of the mouse *in vitro*, acid secretion was decreased. Gray and Wieczerowski (157) showed that after repeated injections, dogs occasionally became refractory to enterogastrone preparations although they continued to manifest depression of secretion when fed fat.

Relation Between Secretory and Motor Inhibition. A final statement cannot be made as to whether or not there are separate physiologic hormones for motor and secretory inhibition. In regard to the physiologic evidence on this question, simultaneous studies correlating motor and secretory inhibition in response to fat or sugar in the intestine have been made only with test meals and here the factors are too complex for satisfactory analysis. The evidence from studies on intestinal extracts is not conclusive either. The motor inhibitor in intestinal extracts is not the physiologic motor inhibitory hormone (see above). The secretory depressant in intestinal extracts may or may not be the physiologic hormone; if it is, then the physiologic hormones for motor and secretory inhibition are separable.

UROGASTRONE

Definition. Urogastrone (153) is the specific principle extractable from the urine of man and animals which upon parenteral administration inhibits the secretion of acid by the gastric glands. Its hormone status is uncertain.

Proof of Existence. Since the definition of urogastrone does not imply a physiologic role for it (although, of course, it may be found to have one) the only steps required to prove its existence are *1*) to show that urine extracts inhibit secretion of acid by the stomach, and *2*) to show that this effect is not due to other substances known to be present in urine, such as hormones or non-specific substances such as pyrogens. These points will be completely covered in the ensuing discussion. The history of the steps leading to the discovery of urogastrone has been recounted by Sandweiss (391).

Physiologic Action of Injected Extracts. Injection of urine extracts has been shown to inhibit gastric secretion in the dog (130, 161, 344), cat (132, 212), rat (133,

378), guinea pig, pigeon (132) and man (160, 398), but not in the frog (161). About 0.1 mg. per kg. of a purified preparation injected intravenously is required to produce 50 per cent or more inhibition of gastric acid output lasting 2 to 3 hours in dogs with gastric pouches receiving histamine subcutaneously every 10 minutes (161). The material is also effective when given subcutaneously, but only at much higher doses; it is not effective by the oral route (161). The relationship between volume and acid concentration during inhibition by urogastrone is the same as that described above for enterogastrone. The effect on pepsin secretion has not been studied. In a series of acute experiments on dogs, Friedman (125) and Friedman and Sandweiss (132) found that the gastric secretory inhibitory effect of urogastrone was low or absent in enterectomized dogs. Schiffrin and Gray (405) reinvestigated this question in semichronic enterectomized gastric pouch dogs and found urogastrone to be fully effective in inhibiting secretion. Urogastrone does not inhibit salivary secretion or urine formation (150, 132), but the secretion of bile and pancreatic juice is somewhat decreased by large doses of urogastrone preparations (161).

Like intestinal extracts, urogastrone preparations contain a motor inhibitory substance (45, 150) which is effective only against the activity of the vagally innervated stomach (204).

Differentiation of Urogastrone from Other Substances in Urine. Purified urogastrone concentrates do not induce estrogenic or gonadotropic changes in immature rats (161, 397). The pressor principle of the posterior lobe of the pituitary inhibits gastric secretion and motility and has been reported to be present in urine. In contrast to pituitrin, however, urogastrone exerts neither pressor nor antidiuretic effects and is relatively less effective in inhibiting gastric motility (154). Urogastrone has been distinguished from kallikrein, a vasodilator substance obtained from urine (161). Urine extracts do not contain prolactin (129).

Pyrogenic substances occur in small quantities in freshly voided urine and are apt to increase markedly if proper precautions for preservation are not taken (158). Unless special efforts are directed toward removal of pyrogens, most urogastrone preparations will contain significant amounts. It is possible to prepare extracts quite free of pyrogens, as evidenced by the fact that such extracts do not produce pyrexia even in doses which are multiples of the gastric secretory inhibitory dose (153, 161). Unfortunately, few investigators have studied the pyrogen content of their urine extracts, so that the interpretation of much of the literature in this field is subject to the reservation that an undetermined portion of the gastric secretory depression may have been due to pyrogen. Although this situation has been known for some time reports of studies on urine extracts (and intestinal extracts) continue to appear in which no measurements of body temperature have been made.

Factors Affecting Excretion of Urogastrone. The possibility that urogastrone is an excretory product of enterogastrone has been examined, but the evidence is inconclusive. Friedman and associates (131) found that gastrectomy and duodenectomy did not depress urogastrone excretion. Preliminary experiments by Culmer and co-workers (74) indicated that complete enterectomy abolished and the feeding of fat enhanced urogastrone secretion but subsequent, more extensive studies by Gray and associates (152) failed to provide complete confirmation for either of these claims.

The latter studies showed *1*) that urogastrone excretion was decreased, but not abolished, by removal of the small intestine, and *2*) that urogastrone excretion was increased over the fasting level by taking in food, regardless of whether the diet was high or low in fat. In the opinion of the reviewer, from the evidence at hand no definite conclusion can be reached concerning whether urogastrone represents an excretory product of enterogastrone. A final decision on this important question must await the application of improved methods which will insure quantitative recovery of urogastrone and removal of nonspecific gastric secretory depressants from the extracts.

Urogastrone has been shown to be present in the urine of pregnant and non-pregnant female human subjects (73, 397) and in male subjects with and without peptic ulcer, gastric carcinoma, or pernicious anemia (130, 134, 159). Gray and co-workers (152) found that urogastrone excretion was significantly lower in patients with peptic ulcer than in normal individuals. This important observation should be confirmed.

Kaulbersz and associates (246–249, 352, 395) have studied the effect of extirpation of various endocrine glands upon urogastrone excretion in dogs. Urine extracts from hypophysectomized and oophorectomized dogs did not inhibit gastric secretion whereas urine from thyroidectomized dogs produced as much inhibition as urine from normal dogs.

Preparation and Chemical Properties. Urogastrone has been prepared from the urine of the dog (153) and man (159). Since urogastrone activity was first demonstrated in urinary extracts containing chorionic gonadotropin, the method for the preparation of chorionic gonadotropin from pregnancy urine has been applied to the preparation of urogastrone from urine of males and non-pregnant females. Various modifications and extensions of the original Katzman and Doisy method for chorionic gonadotropin have been made (161, 196, 398). The purest preparation which has been reported is the material which Gray *et al.* (161) described. It was effective in doses of the order of 0.05 mg. per kg. intravenously and produced no pyrogenic effect. The chemical properties of urogastrone indicate that it is some kind of organic base of considerably less complexity than peptone (161).

Assay. The methods for the assay of urogastrone are the same as those used for enterogastrone and are discussed under that heading.

ANTHELONE

Definition. Anthelone (390) is the principle (or principles) extractable from small intestinal mucosa and from urine which exerts an anti-ulcer effect in Mann-Williamson dogs. Its hormone status is uncertain.

Origin of the Term 'Anthelone' and Justification for its Introduction. The word 'anthelone' is derived from the Greek (anti—against and helckos—ulcer). It was introduced by Sandweiss (390) when it became apparent that the anti-ulcer effect of urine extracts was not due to their gastric secretory depressant (urogastrone) activity. Since a similar situation exists in regard to intestinal extracts, that is, that the anti-ulcer effect is not dependent upon depression of gastric secretion, it is herewith suggested that the term 'anthelone' be applied here too. Since it is not known whether

the anti-ulcer factors from urine and from intestinal mucosa are the same, to distinguish between the anthelones from the two sources the terms 'uroanthelone' and 'enteroanthelone' have been suggested[1] to the reviewer and have the merit of being self-explanatory.

One point requires emphasis in regard to the use of the term anthelone. While it is unquestionably useful to distinguish the anti-ulcer from the anti-secretory action of the intestinal and urinary extracts, it must be remembered that anthelone has not been chemically separated from urogastrone or enterogastrone, and the possibility that a single substance in each of these extracts is responsible for these two kinds of activity must be borne in mind. Urinary and intestinal extracts, prepared in exactly the same manner as in making urogastrone and enterogastrone concentrates, are called anthelone preparations when they are applied to the treatment of peptic ulcer. Anthelone is a designation for an ingredient which has anti-ulcer properties and it is not known whether it is different from the ingredient which gives these extracts antisecretory properties. The use of the term anthelone as though it referred to an extract different from the urogastrone or enterogastrone concentrates, has led to some confusion. Of course, the possibility remains that anthelone may some day be separated from urogastrone and enterogastrone.

The Mann-Williamson Operation. The Mann-Williamson (M-W) operation (322) consists of a gastrojejunostomy with an anastomosis to the terminal ileum of the duodeno-jejunal segment, which contains the bile and pancreatic ducts. Almost 100 per cent of dogs so operated die with a jejunal ulcer opposite the gastrojejunal stoma in an average of about 75 days, when an ordinary kennel diet is fed (386). Prolongation of life and prevention of occurrence of ulcer are the criteria used in assessing the value of treatments.

Enteroanthelone. a) In M-W dogs. Intestinal extracts in the form of enterogastrone concentrates prepared from the small intestine of hogs were first administered intravenously (195) to M-W dogs in doses of 50 mg. three times daily, with the thought that this might inhibit secretion sufficiently to prevent postoperative jejunal ulcer. It turned out that although gastric secretion was not significantly affected, jejunal ulcers developed in only 6 of the 25 dogs during a one-year period of treatment. Ten dogs treated with an extract prepared from pork skeletal muscle showed the same survival and incidence of ulcer as untreated controls. In subsequent studies in Ivy's laboratory (222, 223), it was demonstrated that intestinal extracts were also effective when administered subcutaneously once daily in doses too small to depress gastric secretion. Studies in other laboratories on the effect of subcutaneously injected intestinal extracts (enterogastrone concentrates) on the M-W ulcer gave negative results in two instances (360, 386) and equivocal results in a third (457). All three of these groups used extracts from the same commercial source and it is possible that their failure to confirm the results from Ivy's laboratory was due to the use of an inactive material.

b) In human peptic ulcer patients. Ivy and co-workers (234) used recurrence rate as a criterion of benefit, and found that the majority of the 46 patients receiving enterogastrone concentrates intramuscularly three or six times weekly for one year

[1] These terms were suggested by Dr. A. Littman.

experienced fewer recurrences during and after discontinuation of treatment than before beginning treatment. Sandweiss (399) and Pollard (358–360), using the same enterogastrone concentrate which they had found to be ineffective in M-W dogs, reported little or no benefit from its use in peptic ulcer patients.

Uroanthelone. a) In M-W dogs. Because they had observed amelioration of symptoms in peptic ulcer patients during pregnancy, Sandweiss and co-workers (396) tested on the M-W dog the effect of injection of various endocrine preparations. Among the hormones tested, chorionic gonadotropin, prepared from the urine of pregnant human subjects, was the only one which reduced the incidence of ulcers. Broad and Berman (48) confirmed the beneficial effect of the chorionic gonadotropin preparation. Subsequent studies by Sandweiss' group (386, 389, 393, 397, 398) revealed that urine from non-pregnant women, and therefore devoid of chorionic gonadotropin, also exerted this beneficial effect, whereas urine from normal men was less effective and that from patients with peptic ulcer was stated to be ineffective. Statistical analysis of Sandweiss' data (230) revealed that the difference between the rates of effectiveness of urine extracts from pregnant and non-pregnant females was not statistically significant, nor was that of urine extracts from normal men and male peptic ulcer patients. The difference between urine extracts from females (pregnant and non-pregnant) and males (with or without ulcer) was, however, highly significant. When it was reported that extracts of urine from patients with peptic ulcer inhibited gastric secretion (130), but did not protect against M-W ulcers (386, 389), this was taken as evidence that urogastrone was present and uroanthelone was absent. This finding favors the view that they are separate substances. A later study (152), however, showed that in peptic ulcer patients urogastrone excretion is reduced below normal, and reexamination of Sandweiss' data (230) revealed that anthelone activity was not entirely absent from the urine of these patients; this evidence favors the view that urogastrone and uroanthelone are identical.

b) In human peptic ulcer patients. Only one group of ulcer patients has been treated with injections of urine extracts (398). These patients received small doses intermittently over a short period. Moderate immediate improvement was noted, but no suppression of recurrences. Because of the small doses and the short period of treatment, this cannot be considered as a conclusive measure of the value of this type of therapy.

Phenomenon of 'Immunity' after Cessation of Treatment with Anthelone. After treatment with either uroanthelone (390) or enteroanthelone (230) M-W dogs manifest a marked resistance to development of ulcer, sometimes living for two or three years without further treatment and without developing ulcer. By contrast, when ulcer formation is prevented in M-W dogs by administration of aluminum phosphate gel (108), cessation of treatment results in development of ulcer within two to three months. This indicates that the process responsible for protection against ulcer during treatment with anthelone continues to operate for long periods after its administration has been stopped.

Mechanism of Action of Anthelone. Since it is clear that the doses of uroanthelone and enteroanthelone which have been found to be large enough to be effective in preventing ulcer in a high percentage of M-W dogs are too small to produce significant

inhibition of gastric secretion (195, 394), some other explanation for the mechanism of its beneficial effect must be sought. Prolonged administration of intestinal (182) or urinary extracts (394) to normal or M-W dogs does not lead to depression of the secretory capacity of the gastric glands, so the action of anthelone cannot be attributed to some type of cumulative effect on the gastric glands. The only alteration in secretion which has been observed to result from the administration of enterogastrone concentrates to M-W dogs is a reversion of the excessively prolonged secretory response to alcohol to a normal type of response (182). Whether this is the cause of or is the result of the therapeutic effect is conjectural.

Sandweiss and co-workers (33, 391) noted that in M-W dogs treated with uroanthelone many of the dogs which developed ulcer showed histological evidence of healing of the ulcer, consisting of epithelialization of the base, fibroplasia, and formation of new capillaries. They proposed that the action of anthelone be attributed to this stimulation of the healing process. Since, however, a very significant percentage of M-W dogs treated with uroanthelone or enteroanthelone do not develop ulcer at all, it is questionable whether the healing tendency noted by Sandweiss' group was due to a direct action of the extracts on the intestinal mucosa or merely to a diminution of some influence which promotes ulcer formation.

Tests for Anthelone Action Other than the M-W Dog. Because of the long time required for experiments on M-W dogs, other means of assaying for anthelone activity have been sought. The procedure which has become most popular is the Shay rat test (415) in which the occurrence of ulceration in the rumen of rats after ligation of the pylorus is observed. Certain intestinal (378) as well as urinary (353, 378, 472, 473) extracts have been shown to be capable of inhibiting the formation of these ulcers, but it has also been shown that they accomplish this by depressing gastric acid secretion (378). Thus, there is no reason to believe that this effect is a measure of the same kind of anthelone activity as is responsible for the effect in M-W dogs (333). Furthermore, none of the investigators working with intestinal and urinary extracts in Shay rats have measured the pyrogen content of their extracts, and McGinty and co-workers (325) have shown that highly purified bacterial pyrogens can both suppress secretion and counteract ulcer formation in the Shay rat.

Several procedures based upon an increase in rate of healing of artificially produced defects in the gastric mucosa have been proposed as a test for anthelone action (124, 189). These require confirmation and further study.

Treatment with urinary (105, 336, 417) and intestinal (198) extracts failed to protect against the occurrence of peptic ulcer in dogs receiving cinchophen. Intestinal extracts also failed to prevent ulcers in dogs receiving histamine in beeswax (181). These tests on cinchophen- and histamine-induced ulcers should be repeated when more highly purified extracts become available.

Oral Administration of Urinary and Intestinal Extracts. At the present time oral administration of intestinal and urinary extracts to patients with peptic ulcer is being widely practiced (139, 351, 375, 392). Some have claimed excellent results, but unless a clinical study is rigidly controlled, evaluation of results is difficult. In one excellently controlled clinical study (139), oral administration of intestinal extract was found to be only questionably beneficial.

In regard to oral administration of urinary and intestinal extracts to M-W dogs, although the first report on intestinal extracts—a preliminary report by the author of this review (185)—indicated that they were active orally in preventing ulcers, subsequent studies on both intestinal (358, 385, 386) and urinary (386) extracts have shown little or no benefit from these substances. The intestinal and urinary preparations which have been tested by oral administration are crude extracts unsuitable for parenteral administration. No one has studied the oral effectiveness of a purified extract which has been proven to be active by parenteral administration. Intestinal extracts were found inactive by oral administration when assayed for the chick anti-gizzard erosion factor (162)

DUOCRININ

Definition. Duocrinin[2] (Grossman, 1950) is the hormone which is released by the intestinal mucosa when certain foodstuffs are in contact with it and which stimulates the duodenal glands of Brunner to secrete.

Proof of Existence. Florey and co-workers (117–119, 121, 480) and Fogelson and Bachrach (123) showed that fistulas of the first portion of the duodenum display a secretory response to the feeding of a meal, and this response was shown by Florey and co-workers (119, 480) to persist after transection of the pedicle of a subcutaneously transplanted fistula in the dog, cat, and pig. This has been confirmed for the dog by Sonnenschein and co-workers (423). Florey (121) pointed out that this juice very probably arises from the Brunner's glands rather than from the crypts of Lieberkühn because *1*) fistulas of the lower portions of the intestine, possessing crypts of Liberkühn but no Brunner's glands, produced significant quantities of fluid only after some form of local stimulation and *2*) after stimulation of the duodenal fistula to secrete, for example, by perfusing it with hydrochloric acid, the Brunner's glands showed histologic evidence of depletion of mucus.

The possibility that absorbed secretagogues are the sole humoral agents has been ruled out by demonstrating that oral administration of hydrochloric acid will stimulate secretion in a tranplanted pouch whereas intravenous administration of this acid will not (39).

In further support of the hormonal nature of the process, it has been clearly shown that certain intestinal mucosal extracts upon parenteral injection act as powerful stimulants to the Brunner's glands (6, 7, 120, 123, 423).

It is possible that the discrepancies in the earlier literature concerning the effect of feeding or ingestion of hydrochloric acid on the flow of intestinal juice, some claiming stimulation (47, 78) and others failing to observe it (51), may have been due in part to differences in the levels of the intestine being studied. Some who obtained positive results (78) specified that they used a loop of the lower duodenum and this probably contained enough Brunner's glands to account for the result. A similar explanation may serve to account for some of the discrepancies in the results of experiments on the effect of injection of intestinal extracs on the secretion of intestinal juice, some investigators claiming definite stimulation (78, 43, 137), others failing (122).

[2] This is a new term, used for the first time in this review.

Mechanism of Release. a) Site of formation. No special study of the site of formation of duocrinin has been made either by extraction of the mucosa or by determining the regions where stimuli capable of releasing it are able to act.

b) Stimuli for release. Ponomarev (361) was the first investigator to study the secretion of an isolated pouch of the first portion of the duodenum. He failed to obtain secretion in response to the feeding of a mixed meal, but found that 0.5 per cent HCl inserted into the stomach increased the secretion slightly, while olive oil and 5 or 10 per cent solutions of sodium oleinate increased the secretion markedly. In similar experiments on the cat Florey and Harding (120) observed no stimulation of secretion when olive oil or cane sugar was fed but a definite response to a mixture of meat and milk, and a smaller response to 0.1 N HCl. The most extensive studies on the response to various foodstuffs have been those of Blickenstaff and co-workers (40). They observed a definite secretory response to a mixed meal, as well as to test meals of dextrose, cottonseed oil, or lean beef. Saline as a control test meal produced no response.

Role of Motility in Producing the Secretary Response. When Fogelson and Bachrach (123) demonstrated that SI (Greengard and Ivy's crude secretin preparation) stimulated Brunner's gland secretion, they observed that it also stimulated the motor activity of the duodenum, and suggested that the secretory response might be the result of mechanical stimulation caused by the rubbing together of the mucosal surfaces during contractions (see sections on CCK and motor hormone). Evidence that such a mechanism actually operates was gained by Blickenstaff and co-workers (39, 40). These investigators used two types of animal preparations. In the first or pouch type the segment of intestine was left as a tube in the usual fashion. In the second or flap type the segment was cut lengthwise along the antimesenteric border and sutured to the abdominal wall as a rectangular flap. Distinct differences in the secretory responses of these two types of preparation were observed. Feeding of concentrated dextrose solutions or injection of a parasympathomimetric drug produced a secretory response only in the pouch and this was accompanied by increased contractions, whereas intravenous injection of SI or placing of hydrochloric acid in the stomach produced a secretory response in both the flap and the pouch. These findings are preliminary and require confirmation but they suggest that there may be two separate hormonal factors regulating Brunner's glands, both apparently present in SI, one acting as a direct secretory stimulant, the other stimulating secretion by inducing contractions.

Preparation and Properties. Up to the present, the only types of intestinal extracts which have been studied as stimulants of Brunner's glands are secretin preparations. Florey and Harding (120) used Mellanby's purified secretin and Ågren (6, 7) used his crystalline secretin picrolonate and both concluded that secretin is the hormone stimulating Brunner's glands. Sonnenschein and co-workers (423), however, using Greengard and Ivy's cyrstalline secretin picrolonate (see section on SECRETIN) observed no stimulation of Brunner's gland, and more recently Blickenstaff and Grossman (39) have found that highly purified commercial secretin (Lilly) fails to stimulate although it calls forth abundant pancreatic flow. It may be concluded, therefore, that the stimulation of Brunner's glands by certain secretin preparations

is not due to the secretin they contain but to some other specific constituent. For this reason the name 'duocrinin' is here proposed to designate this constituent. Preparations of duocrinin free of secretin have not yet been made.

ENTEROCRININ

Definition. Enterocrinin (337) is the hormone which is released by the intestinal mucosa when appropriate stimuli are in contact with it and which increases the volume rate of secretion and the enzyme output of succus entericus by the jejunum and ileum.

Proof of Existence. The facts which support the existence of enterocrinin are the demonstrations by one group of workers (338) *1*) that, in dogs, subcutaneously autotransplanted loops of jejunum and ileum show an increase in enzyme output several hours after feeding a mixed meal (340, 406) and *2*) that intravenous injection of appropriately prepared intestinal extracts causes an increase in rate of secretion and in enzyme output from jejunal and ileal loops (337).

Certain considerations prevent the acceptance of enterocrinin as an established physiologic entity: *1*) The augmentation of enzyme output in response to feeding occurred only after extrinsic denervation of the jejunal or ileal loop (340, 406) thus indicating that the mechanism does not operate under normal physiologic conditions and thereby raising the question of its physiologic significance. Earlier workers had also found an increase in volume of secretion after denervation (47, 330, 402). *2*) The increase in volume rate of secretion of transplanted jejunal loops noted in the earlier studies from Nassett's laboratory (44, 340) was not found in the animals used in the last study reported by these workers (406). No explanation for this discrepancy was offered by these workers. *3*) Only two dogs with ileal loops have been reported upon by Nasset's group (406) and neither of these showed an augmented volume of secretion in response to feeding either before or after transection of the pedicle, whereas injections of enterocrinin preparations produced an augmentation of both volume rate and enzyme output from both jejunal and ileal loops. This, together with the point mentioned in *2* above, indicates an important lack of similarity between the endogenous humoral agent and the intestinal extract. *4*) The possible role of absorbed secretagogues has not been fully clarified. Nassett and Pierce (339) showed that certain peptone preparations when given either orally or intravenously were effective in stimulating jejunal secretion. They also showed that the active ingredient in peptone was susceptible to inactivation by ereptic and tryptic digestion, whereas the active principle in intestinal extracts was not. *5*) The possible role of stimulation of motility in producing increased volume or enzyme output has not been adequately investigated (see duocrinin). The fact that most of the enzymes of intestinal juice are endocellular in type and probably not an exocrine secretion (480) suggests that the stimulation of enzyme output is likely to be the result of mechanical stimulation by increased motor activity.

For these reasons further work is necessary before enterocrinin can be included with the established gastrointestinal hormones.

Mechanism of Release. a) Site of formation. The effect of food is presumed to take place in the intestine although no experiments have been performed in which passage through the stomach is eliminated. Nassett found enterocrinin activity in

extracts from both the small intestinal and colonic mucosa, but none from that of the stomach, liver, pancreas, kidney or skeletal muscle (337).

b) Stimuli for release. A mixed meal has been the most frequently studied stimulus (340, 406). When fed to dogs with jejunal transplants, certain kinds of peptone were found to be effective whereas others were not (339). Meals of potato or beef heart have also been shown to be moderately effective stimuli (44).

Preparation and Chemical Properties. The basic method for extracting enterocrinin preparations from intestinal mucosa consists of extraction of the intestines with slightly acidic aqueous ethyl alcohol, removal of the alcohol and salting out with NaCl. Extensive studies on purification have been made (114).

Enterocrinin was found in the mucosa of the hog, dog, cat, human, monkey and cow (337). Highly purified preparations were effective in doses as low as 36 μg. intravenously (114). Extracts were also effective when given subcutaneously but much larger doses were required by this route (406).

Purified preparations had no secretin (114, 337) or enterogastrone (406) activity and were free of vasodilatin. Unfortunately the effect on gastrointestinal motility has not been determined.

Assay. Nasset and co-workers (115) used a cannulated loop of jejunum in anesthetized dogs, and measured activity of the preparations in terms of increase in rate of flow of juice over control levels. The temperature and the intralumenal pressure of the jejunal loop were controlled.

THE QUESTION OF A HORMONE FOR INTESTINAL MOTILITY

All who have studied the phenomenon (28, 58, 87, 213) agree that the small intestine of an animal exhibits an increase in motility within a few minutes after feeding. This effect occurs when food is admitted to the stomach through a gastrostomy and it is not abolished by vagotomy. Most investigators have found that any interruption of the continuity of the intestine, such as in a Thiry or Thiry-Vella loop, abolishes the augmentory effect of feeding even though the extrinsic nerves to the loop remain intact (87, 214, 238, 369). Puestow (367) and Raiford and Mulinos (374), however, stated that isolated segments of jejunum and ileum showed a delayed motor response to feeding and that this response was abolished by transection of the pedicle. Regardless of this discrepancy in the observations on innervated isolated loops, there is no published report of augmentation of contractions in response to feeding in transplanted intestinal loops, although it must be added that none of the investigators working with transplanted loops of intestine have presented detailed data on this point. Recently, in the reviewer's laboratory evidence of augmentation of motility as a result of feeding, in dogs with transplanted segments of the first part of duodenum, has been obtained (39) (also, see section on DUOCRININ). These observations are not yet extensive enough to be conclusive.

The existence in intestinal mucosal extracts of a factor stimulating intestinal motor activity has been recognized by several investigators (27, 103, 126, 319, 388, 482) (see also under CCK). However, until such time as a humoral mechanism for control of intestinal motility has been demonstrated, no physiological role should be assigned to these substances.

It has been observed a number of times that the injection of crude intestinal

extracts ('secretin concentrates') into experimental animals results in a transitory but definite increase in red blood cell count (88, 138, 256, 284). This has been clearly demonstrated to be due to contraction of the spleen (113, 284) and not to formation of new cells because it is unaccompanied by reticulocytosis (112, 256). The spleen-contracting substance has been differentiated from histamine, choline, acetylcholine, and secretin (113). Presumbly it is the same substance which stimulates intestinal motility. Its relationship to cholecystokinin is an important unanswered question.

Considering all the evidence available, it can be concluded that there are no convincing physiologic studies which suggest the existence of a motor hormone for the intestine. On the other hand the possibility of the existence of such a hormone has not been ruled out.

VILLIKININ

The intestinal villi show marked motor activity when chyme is in contact with them. The early investigators emphasized the importance of local chemical and mechanical stimuli for evoking these movements, since they occurred only in intestinal segments to which chyme had descended (254, 263). In 1933, however Kokas and Ludány (264, 265) introduced the concept of a hormonal control by demonstrating that the introduction of hydrochloric acid into the duodenum would activate the movements of the villi of a jejunal segment which had been removed from the abdominal cavity and which was being viviperfused by anastomosis with the external jugular vein and the carotid artery. This observation was subsequently confirmed by the same investigators (266) in experiments with cross circulation between two dogs. Crude preparations of secretin were shown to stimulate movements of the villi (268) and the active principle, which was shown to be distinguishable from histamine, choline, adenosine, secretin and cholecystokinin, was given the name 'villikinin' (267). The distribution and chemical properties of villikinin have been studied rather extensively (265, 269, 311–314). Villikinin was shown to be effective when placed in the lumen of an isolated intestinal loop, and was thus considered to be absorbable (270). Kokas and Ludány (271) reported that the addition of dilute HCl to glucose solutions increased their rate of absorption from the intestine, presumably by eliciting the villikinin mechanism, but Loew, Gray and Ivy (307) were unable to confirm this effect of HCl upon glucose absorption (see section on hypoglycemic action of duodenal extracts). All of the work on villikinin has come from Kokas and Ludány's laboratories; no confirmation from other laboratories has yet appeared.

MISCELLANEOUS TOPICS

Hormonal Control of Pepsin Secretion. Evidence has been brought forth, both from physiologic experiments and from injection of extracts, that the intestinal mucosa or the pyloric mucosa (or both) may produce a hormone which stimulates the secretion of pepsin. Friedman and associates (128) found that in Pavlov and Heidenhain pouch dogs the instillation of hydrochloric acid into the duodenum increased the pepsin content of the gastric secretion in response to a meal or to insulin but not to histamine. Pratt (365) injected crude secretin into cats and found that it simulated pepsin secretion. It was subsequently shown that highly purified secretin did not have

this pepsigogic property (23, 52) and that the active principle was present in crude pancreozymin concentrates (470).

Savich (403) and Zeljony and Savitch (481) claimed that distention of the pyloric part of the stomach, in addition to stimulating the secretion of acid by the fundic glands (see GASTRIN) also acted as a powerful stimulus for pepsin secretion. Recently Uvnäs (452) has reported one experiment in a dog which he interprets as confirming Savich. Slezak and Grossman (416) have investigated this problem extensively and have found that the peptic power of gastric juice secreted in response to distention of the pyloric part of the stomach is not significantly different from that of gastric juice secreted in response to injections of histamine, provided the volume of juice resulting from the two stimuli is comparable. This confirms an earlier study in which it was shown that pepsin concentration of gastric juice secreted by a transplanted fundic pouch was on the same low level when stimulated by perfusion of liver extract through the main stomach as when stimulated by injecting histamine, again provided the volumes of secretion were comparable (191). Uvnäs (449, 452) reported that certain extracts of pyloric mucosa prepared according to his method for recovering gastrin caused an increase in pepsin concentration, and that the pepsigogic effect was not correlated with the acid stimulating potency.

The general problem of hormonal regulation of zymogen secretion has been reviewed by Thomas (433).

In summary, the evidence for the existence of a hormone from the intestinal or pyloric mucosa for the regulation of pepsin secretion is no more than suggestive.

Production of Hypoglycemia by Administration of Duodenal Extracts. Numerous investigators have reported that various types of intestinal extracts, especially those of duodenal mucosa, upon injection or even ingestion, are capable of reducing the blood sugar concentration. The active principle of these extracts has been variously named 'incretin,' 'duodenin,' and 'insulotropic hormone,' (306–308, 285). Apparently it was this purported hypoglycemic action of intestinal extracts which led some investigators to search for physiologic evidence which would support the theory that a duodenal hormone is concerned in carbohydrate metabolism. Several workers have reported success in their attempts to demonstrate that the presence of hydrochloric acid in the duodenum lowers the blood sugar level, although some investigators have maintained that this can be demonstrated only in the absence of the adrenal glands.

Recently, Loew and co-workers (306–308) have comprehensively re-studied this problem, using large numbers of animals in each phase of the study and employing rigid control procedures. They found that the presence of hydrochloric acid in the duodenum failed to lower the fasting blood sugar level of normal anesthetized or unanesthetized dogs, or of dogs with ligated adrenal glands. Acid in the duodenum also failed to reduce the hyperglycemia resulting from intestinal absorption of glucose, injection of epinephrine, or removal of the pancreas. Finally, Loew and co-workers (308) prepared extracts of intestinal mucosa by a variety of methods which have been reported to yield the hypoglycemic substance but none lowered the fasting blood sugar level of trained, unanesthetized dogs.

Until more convincing evidence has been presented for the hypoglycemic activity of intestinal extracts, or for the effect of duodenal stimulation on blood sugar

level, the existence of a duodenal hormone for the regulation of sugar metabolism must be considered questionable.

CONCLUDING REMARKS

The only two gastrointestinal hormones which may be considered to be fully established both by demonstration of endogenous release physiologically and by demonstration of the activity of mucosal extracts are secretin and cholecystokinin. For gastrin, pancreozymin and enterogastrone the evidence is strong but not conclusive. The physiologic demonstration of gastrin is conclusive but the work on extracts requires further confirmation and extension. In the case of pancreozymin both the physiologic and tissue extract studies are convincing but require further confirmation. For enterogastrone the physiologic studies are conclusive but the motor inhibitory principle has not yet been demonstrated in tissue extracts. Urogastrone and anthelone are biological extracts and no physiologic role for them is yet evident. Duocrinin has both physiologic studies and tissue extract work supporting its existence but these require confirmation. The physiologic evidence for enterocrinin is inconclusive and the work with extracts is unconfirmed. Both phases of the studies on villikinin require confirmation. There is only suggestive evidence for the existence of other gastrointestinal hormones.

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BIOCHEMISTRY OF MELANIN FORMATION

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MELANIN PIGMENTATION has aroused the curiosity and attention of man since the beginning of recorded history. This interest stems in part from the social, protective and cosmetic significance of pigmentation. In addition, much interest has arisen concerning the possible role of melanin formation in the development of the melanoma which has been described as having the most sinister reputation of malignant tumors (1). As Ewing (2) has stated, "The variations in pigment offer a very delicate indication of the functional activity of the cells and a unique opportunity to trace the relation between the functional activity and the growth capacity of the tumor cells." As a result, this subject has attracted the attention of workers in many fields (3), for example, the cytologist in the study of the melanin granules; the geneticist, the anthropologist and the biologist in a study of the relationship of heredity, environment and nutrition to the development of pigment; and the organic and biologic chemists in the study of the chemical mechanism underlying melanin formation.

In the past quarter of a century several comprehensive reviews on the mechanism of melanin formation have appeared (4–11), but in only a few of these has there been any consideration of the biochemical aspects of this problem. No attempt was made to connect the processes of melanin formation operating in various species. Different mechanisms were proposed for humans, lower animals and plants.

Investigation of the biochemistry of melanin formation in plants, insects and marine animals had shown that the enzyme *tyrosinase* catalyzes the oxidation of L-tyrosine to dihydroxyphenyl L-alanine (dopa) and then the oxidation of dopa to melanin.

$$\text{Tyrosine} \xrightarrow{\text{Tyrosinase}} \text{Dopa} \longrightarrow \text{(Intermediates)} \longrightarrow \text{Melanin}$$

Until recently, however, the presence of tyrosinase in mammalian tissue had not been conclusively demonstrated, and it was believed that melanin in mammalian tissue is formed by a mechanism different from that operating in other species. Histochemical evidence indicated that mammalian skin contains an enzyme, *dopa-oxidase*, which catalyzes the oxidation of dopa, but not tyrosine, to melanin.

$$\text{Dopa} \xrightarrow{\text{Dopa Oxidase}} \text{(Intermediates)} \longrightarrow \text{Melanin}$$

[1] Fellow in Dermatology and Syphilology, Mayo Foundation.

Largely as a result of these beliefs, two separate hypotheses of melanogenesis evolved. Melanin formation in insects and plants was associated with tyrosinase, while melanin production in mammalian skin was associated with dopa-oxidase.

In the present review, it is shown on the basis of recent evidence that these separate concepts can now be merged into a single hypothesis to account for melanin formation in man, lower animals, insects and plants. The properties of the enzymes concerned with melanin formation and the mechanisms of their reactions are considered primarily. Attempts will be made when possible to correlate these properties and reaction mechanisms with normal and pathologic melanin pigmentation.

In view of the widespread occurrence of melanin pigment in nature and the keen interest shown in the problem of melanogenesis, it may seem surprising that there has been so much delay in clarifying the mechanism of melanin formation. Several obstacles have impeded progress in research on melanogenesis. Firstly, melanin (a darkly colored polymerized product of the oxidation of *o*-dihydroxyphenyl compounds, such as dopa, epinephrine, catechol) is of unknown chemical composition. Secondly, it is difficult to purify melanin and as a result, different chemical compositions have been reported by different investigators. Thirdly, epidermal extracts could not be prepared which conclusively demonstrated enzymatic activity toward tyrosine and dopa. This was due either to the small quantity of enzyme in the skin or to the fact that extracts made by grinding and homogenizing skin had not been satisfactory. Finally, the formation of melanin from its precursors is a complex process involving several steps, and the rates of the reactions depend on many surrounding chemical and physical factors.

HISTOCHEMICAL STUDIES

As early as 1901 v. Fürth (12) advanced the hypothesis that melanin formation is the result of the action of an intracellular oxidase on aromatic or chromogen groups in certain protein molecules. Bloch, a Swiss dermatologist, stimulated by this hypothesis attempted to prove it by experimental methods (4). His approach was directed by two clinical observations. First, melanin pigmentation is a prominent feature of Addison's disease, a disorder resulting from hypofunction of the adrenal glands. The possibility arose that there might be some relationship, albeit a paradoxical one, between the increased pigmentation and the metabolism of epinephrine-like substances. Second, in cases of metastatic melanoma with melanuria, the urine contains significant amounts of catechol derivatives. These facts suggested to Bloch a chemical similarity between the precursor of melanin and the compounds epinephrine and catechol. He therefore selected a naturally occurring amino acid, 3,4-dihydroxyphenyl-L-alanine[2] (which he abbreviated as *dopa*, using the initial letters of its German name, *di-oxy-phenyl-alanine*) as the substrate for histochemical studies.

Bloch immersed frozen sections of pigmented human skin in a 1:1,000 solution of dopa buffered to *p*H 7.3 to 7.4. He noted that after 24 hours at room temperature melanin granules were deposited in the cytoplasm of cells located in the basal layer

[2] Dopa is found in the wing coverings of cockchafers *Melolontha melolontha L.* and *M. hippocastani F.* (13), and in the cocoons of one of the Saturnidae, *Samia cecropia L.* (14). It also occurs in *Vicia faba* (15) and the Georgia velvet bean, *Stizolobium deeringianum* (16).

of the epidermis. These specialized cells, which Bloch called melanoblasts, are located at the epidermo-dermal junction and were considered by Bloch to be the natural site of melanin formation. The intensity of the response, moreover, corresponded to the known capacity of the skin to form melanin. Melanin deposition was most intense in the areas of the skin capable of heavy pigmentation such as the basal layer of the epidermis, the germinal layer and cells of the hair matrix, and the cells of pigmented nevi. Melanin deposition did not occur in albino skin and in the skin of patients with vitiligo, a skin disease characterized by localized areas of complete loss of melanin pigment. Bloch (4), therefore, concluded that the 'dopa reaction' is a reliable indicator of the capacity of cells to form pigment.

The origin of the melanoblast has been a subject of controversy for many years. Bloch (4), Peck (17) and others have supported the thesis that the melanoblasts are derived from ordinary palisade basal cells under certain types of stimuli. Masson (18) and Becker (19), however, believe that the melanoblasts are of neuro-epithelial origin. In recent years several groups of investigators have carried out embryological studies in amphibia (20, 21), birds (22–24) and mice (25–27) and have shown conclusively that the melanoblasts are derived from the neural crest region.

Further investigation provided evidence that the catalytic effect of certain cells on the oxidation of dopa to melanin is due to the presence of an enzyme, which Bloch called *dopa-oxidase*. The evidence for the enzymatic nature of the dopa reaction included the following: *1*) The reaction did not occur after the tissue had been heated to 100°C.; *2*) a definite *p*H range was required, the optimal *p*H being 7.35; *3*) the reaction was completely inhibited by M/2,000 hydrogen sulfide or M/500 potassium cyanide, which are known inhibitors of some enzymes; *4*) only the levorotatory form of dopa was catalytically oxidized by the cells.[3]

To substantiate further the enzyme hypothesis Bloch and Schaaf (28) prepared extracts of skin from newborn rabbits and demonstrated by visual colorimetric methods the presence of a heat-labile, cyanide-sensitive catalyst capable of accelerating the formation of melanin from dopa. Extracts of albino skin under the same conditions were ineffective in catalyzing the oxidation of dopa. Although Bloch's technics for measuring enzymatic activity were not detailed his data provided convincing evidence that the catalytic effect of certain epidermal cells on the oxidation of dopa is due to the presence of an enzyme.

The presence of dopa-oxidase in normal human or animal skin has not been conclusively demonstrated by enzymatic technics such as the manometric measurement of oxygen uptake by skin slices or extracts in the presence of dopa. Although this has been attempted by several investigators, none has been successful because of the small quantity of enzyme present in the skin as well as the difficulty of preparing skin extracts and homogenates suitable for measuring enzymatic activity. It is also possible that in preparing extracts of skin the investigators neglected to take into account the possibility of a naturally occurring inhibitor which could mask the presence of the enzyme. Recently, Rothman and co-workers (29) succeeded in

[3] Many other related compounds, such as D-dopa, tyrosine, phenylalanine, *p*-hydroxyphenylpyruvic acid, homogentisic acid, quinol, pyrogallol, catechol, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, epinephrine, trihydroxyphenylalanine, glycyl-dopa, pyrrole and tryptophane, did not form melanin granules after incubation with frozen sections of skin.

demonstrating the presence in human epidermis of a water-soluble, dialyzable, heat-stable inhibitor of plant tyrosinase. This inhibitory principle was counteracted by iodoacetamide and another powerful sulfhydryl inhibitor, *p*-chloromercuribenzoic acid. Later work (30) showed a direct relationship between the sulfhydryl group concentration and the inhibitory power of the epidermal extracts.

Bloch's histochemical studies with dopa, which have been amply confirmed, have not been generally accepted as a complete explanation for the mechanism of melanogenesis since dopa has never been demonstrated in mammalian tissue. Raper (31) was able to isolate dopa in crystalline form as the first oxidation product resulting from the action of tyrosinase obtained from mealworms (*Tenebrio molitor*) on tyrosine. This strongly suggested that the reactions in the formation of melanin that are subsequent to dopa production are the same whether the initial substrate is tyrosine or dopa and emphasized the gap in the knowledge of the mechanism of the conversion of tyrosine to dopa in various tissues. Raper (10) provided conclusive evidence that tyrosine, a naturally occurring amino acid present in all tissues including skin, is the initial substrate in the formation of melanin by plant and insect tyrosinase. As stated previously, Bloch's dopa-oxidase reaction could not be obtained after incubation of the frozen skin sections with tyrosine under the same conditions in which dopa was effective. Since it was generally felt that tyrosine is the natural or physiologic substrate in the enzymatic formation of melanin by mammalian tissues as well as by plants and insect tissues, and since the reaction whereby tyrosine is converted to dopa in mammalian cells was not known, the mechanism of melanin formation in mammals remained a puzzling problem for many years.

Much investigation remains to be done on this problem. Bloch's qualitative studies might well be extended by use of present-day microchemical methods for quantitative determinations and histologic localizations of small quantities of enzymes. In addition, through the use of C^{14}-labeled tyrosine it may be possible to show conclusively whether or not tyrosine is the natural substrate involved in melanin formation by melanoblasts.

BIOCHEMICAL STUDIES

Melanin Formation in Lower Animals—the Tyrosinase Concept

The enzymatic nature of the melanin-producing reactions was first satisfactorily investigated in plants and fungi. In 1895 Bourquelot and Bertrand (32) reported that a substance present in the mushroom, *Russula nigricans*, was converted into a black pigment. This substance, later shown to be tyrosine, was acted on by an enzyme in the fungus which was named *tyrosinase* (33). Since that time tyrosinase has been found in a wide variety of plant and animal tissues (34–36) and a vast literature has accumulated on this subject.

Working with tyrosinase obtained from plants and mealworms, Raper (10) was able to determine many of the reaction mechanisms whereby tyrosine is converted into melanin. He showed that in the presence of tyrosinase and oxygen, tyrosine is first oxidized to dopa, and the dopa is then oxidized to dopa-quinone. Dopa-quinone is converted to an indole derivative which, after undergoing several

reactions, polymerizes to form the pigment, melanin. The details of the chemical reactions concerned in this process (fig. 1) will be considered later.

The literature contains several early reports on the occurrence of tyrosinase in mammalian tissue. In 1903, Gessard (37) found that extracts from a horse melanoma were able to catalyze the conversion of tyrosine to melanin. This was confirmed by De Coulon in 1920 (38). In 1907, Alsberg (39) prepared from a hunan melanoma an extract which catalyzed the formation of black pigment from catechol and possibly from tyrosine. In the following year, Neuberg (40) showed that dilute extracts from a human melanoma accelerated pigment formation from tyramine and epineph-

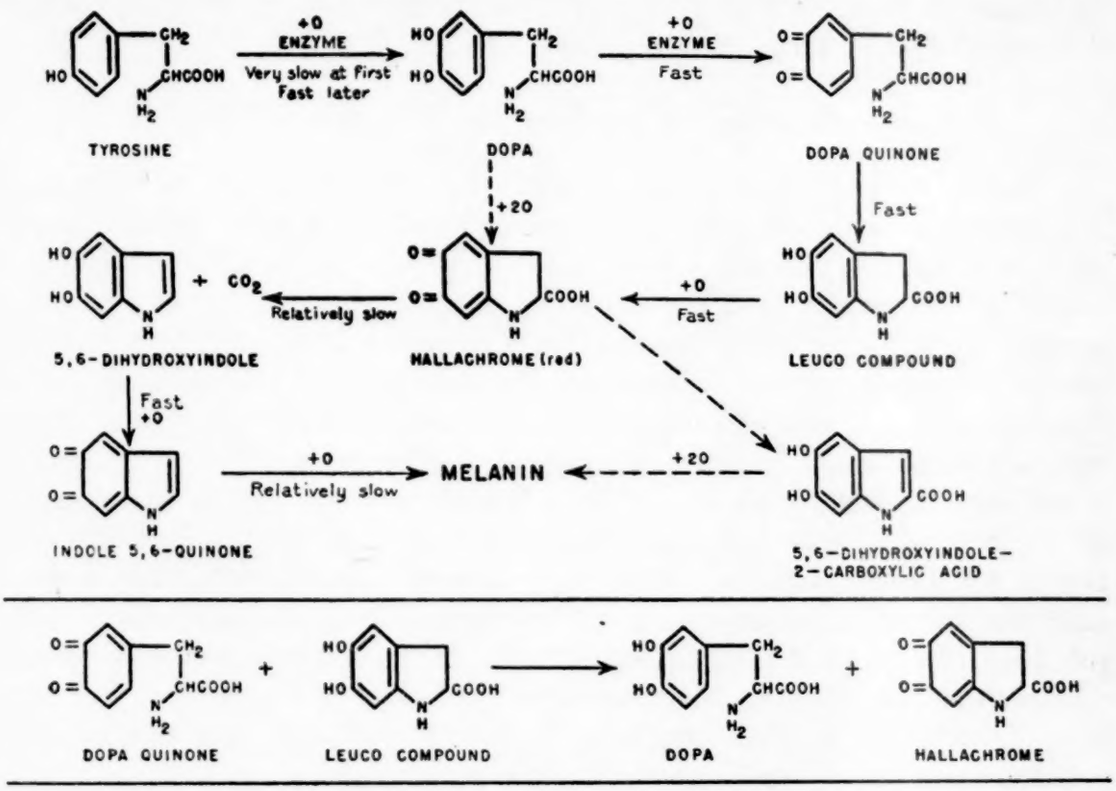


Fig. 1. ENZYMATIC oxidation of tyrosine to melanin.

rine but not from tyrosine. Winternitz reported in 1919 (41) that an enzyme present in the uvea of the hog and the black cutaneous tissue of the horse could catalyze the darkening of tyrosine.

Melanin Formation in Mammals—the Dopa-Oxidase Concept

Despite the early widespread beliefs that tyrosinase occurred in mammalian tissue, plus experimental evidence in accord with these beliefs, skepticism arose concerning the presence of the enzyme in such tissue. For example, the work of Durham (42) on tyrosinase in fetal rabbit skin could not be confirmed (43). In addition, no one was able to demonstrate tyrosinase activity in normal pigmented mammalian tissue. Finally, Bloch (4) as discussed previously, showed that an enzyme present in mammalian skin could catalyze the oxidation of dopa but not tyrosine to melanin. Largely as a result of this work, the concept arose that *dopa-oxidase*, but not *tyrosin-*

ase, was present in mammalian tissue. Although Bloch's work did not pass unchallenged, it remained the most acceptable until recently.

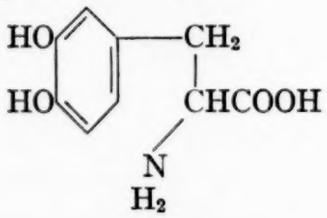
Hogeboom and Adams in 1942 (44), Greenstein and co-workers in 1944 (45, 46) and Lerner, Fitzpatrick, Calkins and Summerson in 1949 (47) showed conclusively that extracts from mouse, human and horse melanomas contain both tyrosinase and dopa-oxidase activities and that these activities are similar to those found in extracts from plants and lower animals. Calkins (48) demonstrated that extracts from normal beef ciliary bodies possess tyrosinase and dopa-oxidase activities. In view of this experimental evidence of the presence of tyrosinase in mammalian tissue, it became necessary to modify the hypotheses of pigmentation which had evolved from Bloch's dopa-oxidase studies.

Chemical Reactions in the Conversion of Tyrosine to Melanin

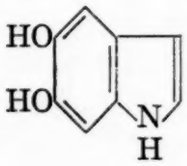
The reactions involved in the enzymatic oxidation of tyrosine to melanin are shown in figure 1. In the presence of tyrosinase and molecular oxygen, tyrosine is oxidized to dopa. This reaction is usually slow at the onset, but after an induction period it becomes very fast. The conversion of tyrosine to dopa is not a reversible reaction. Dopa formed in the first reaction is oxidized enzymatically by a reversible reaction to dopa-quinone. Further stages of the reaction proceed rapidly in the absence of the enzyme although the reaction rates are increased in the presence of the enzyme. Dopa-quinone undergoes a spontaneous irreversible and rapid intramolecular change in which the nitrogen of the side chain attaches itself to the 6-position of the benzene nucleus with the formation of 5,6-dihydroxydihydroindole-2-carboxylic acid (leuco compound). The leuco compound is readily oxidized by a reversible reaction to the corresponding quinone (hallachrome). Hallachrome[4] is a red substance, and it is the first visible product formed in the reactions. Under physiologic conditions hallachrome decarboxylates and undergoes a rearrangement to form 5,6-dihydroxyindole. The indole compound is rapidly oxidized to the corresponding quinone which has a purple color. The quinone then polymerizes to melanin with the consumption of approximately one atom of oxygen. Relatively little is known of the mechanism of this polymerization (49, 50). If the intramolecular rearrangement undergone by hallachrome is quickened by sulfurous acid, no decarboxylation occurs and 5,6-dihydroxyindole-2-carboxylic acid is formed. This latter substance is readily converted to a melanin substance. In the series of reactions shown in figure 1, possible alternate mechanisms are described by broken arrows. Much of the knowledge of the chemical reactions which take place in the enzymatic oxidation of tyrosine has been obtained through the brilliant work of Raper and his co-worker (10, 52a) with potato and mealworm (*Tenebrio molitor*) tyrosinase. They were able to show that the following three substances were formed during the tyrosine-

[4] Hallachrome occurs naturally in the polychaete worm, *Halla parthenopala* (51). Friedheim (52) found that this red substance could accelerate oxygen consumption by erythrocytes and serve as a hydrogen acceptor for xanthine oxidase and succinic dehydrogenase. He suggested that hallachrome may play a role in cellular respiration.

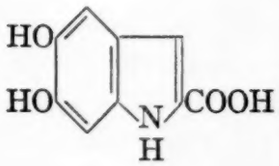
tyrosinase reaction:



I, Dopa



II, 5,6-Dihydroxyindole



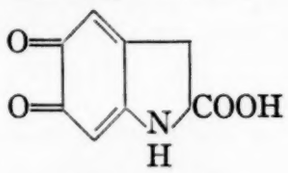
III, 5,6-Dihydroxyindole-2-Carboxylic Acid

When the enzyme is allowed to act on dopa, *substances II* and *III* are formed. This suggests that dopa is probably the first compound formed in the oxidation of tyrosine.

Dopa formed from tyrosine appears to be oxidized to dopa-quinone. The following facts make this plausible: *1*) *o*-dihydroxyphenyl compounds are readily oxidized to the corresponding orthoquinones; *2*) substances which react with orthoquinones inhibit melanin formation in the dopa-tyrosinase reaction (53); *3*) plant tyrosinase has been shown to catalyze the oxidation of catechol to orthobenzoquinone (54). The oxidation of dopa to dopa-quinone would appear to be a similar reaction.

Raper (55) also showed that a red substance (hallachrome) was formed in the oxidation of dopa. This red substance could be reduced to 5,6-dihydroxydihydroindole-2-carboxylic acid (leuco compound) (52a), and the leuco compound so produced readily oxidized back to the red substance. From this it seems likely that dopaquinone undergoes an intramolecular change, the nitrogen of the side chain attaching itself to the 6-position of the benzene nucleus with the resultant formation of the leuco compound.

Since the indole *compounds II* and *III* could be formed from the oxidation of the hallachrome, and since hallachrome was formed from the leuco compound, it was believed that the hallachrome was simply the quinone of the leuco compound and therefore should have the following structure:



Hallachrome

Mason has presented spectrophotometric evidence to support this view (49).

Under normal conditions in the enzymatic oxidation of tyrosine, hallachrome is converted, by decarboxylation and rearrangement, to 5,6-dihydroxyindole. Spectrophotometric data (49) indicate that the 5,6-dihydroxyindole is then rapidly oxidized to the corresponding quinone and the quinone then polymerizes to melanin.

It should be pointed out that the reactions given in figure 1 merely represent the over-all scheme by which tyrosine is converted to melanin. Actually, many more reactions probably occur, such as those involving the formation of semiqui-

nones. The semiquinones may then be oxidized to quinones or undergo rearrangements in accordance with the general picture given in the diagram.

As previously stated, enzyme action is definitely required for the first reaction, the oxidation of tyrosine to dopa. Under physiologic conditions, that is, *p*H 7 to 7.4, the rate of oxidation of dopa to dopa-quinone is fairly rapid without the enzyme but is increased appreciably in the presence of the enzyme. The subsequent reactions shown in the diagram take place rapidly without the enzyme, but even in these cases there is evidence that the presence of tyrosinase will increase the rate of reaction (49).

In addition to the series of reactions discussed above, there is an important interplay of reactions occurring during the conversion of tyrosine to melanin as shown in the lower part of figure 1. Using mealworm tyrosinase, Evans and Raper (52a) found that in a tyrosine-tyrosinase reaction which has proceeded for two to five hours, dopa can be isolated in yields varying from 10 to 20 per cent of the actual tyrosine oxidized, in spite of the fact that this tyrosinase can oxidize dopa more readily than tyrosine. Since the conversion of dopa-quinone to leuco compound does not appear to be a reversible reaction, it seems reasonable to explain the accumulation of dopa on the basis of the presence of some reducing agent or system which reduces the dopa-quinone back to dopa. The oxidation-reduction potentials of the systems concerned in melanin formation in so far as they have been investigated (56) are as follows:

$$\text{Dopa} \rightleftharpoons \text{Dopa-Quinone}$$
$$E_0 = +0.511 \text{ V at } p\text{H } 4.6$$

$$\text{Leuco Compound} \rightleftharpoons \text{Hallachrome}$$
$$E_0 = +0.170 \text{ V at } p\text{H } 46.$$

From these data and from the observation that addition of leuco compound to a tyrosine-tyrosinase system increases the accumulation of dopa, it seems likely that the interplay of reactions shown in figure 1 occurs in the tyrosine-tyrosinase reaction. The importance of this reaction will be discussed later. It is possible that interactions involving substances such as dopa-quinone and 5,6-dihydroxyindole also occur. These reactions, if present, could play important roles in regulating the rate of melanin formation.

Induction Period in the Oxidation of Tyrosine

There are several points in the oxidation of tyrosine that are of great importance and interest. When tyrosine and tyrosinase are allowed to react in the presence of oxygen, there is often a lag period before oxidation of tyrosine begins. This lag interval is referred to as the *induction period* (47). Small amounts of dopa are very effective in shortening the induction period in the tyrosine-tyrosinase reaction (fig. 2). If the induction period is defined as the intercept on the time axis of an extension of the slope of the oxidation curve when oxidation is proceeding maximally, there is for mammalian tyrosinase a linear relationship between the negative logarithm of the dopa concentration and the induction period (47).

Recent studies (57, 58) with mouse melanoma tyrosinase indicate that compounds related structurally to dopa, for example, epinephrine, catechol, and so

forth, can shorten the induction period, but not nearly as effectively as dopa. On an equimolar basis, DL-dopa is about 75 per cent as effective as is L-dopa in shortening the induction period. For mammalian tyrosinase, dopa is a fairly specific catalyst regulating the induction period.

When dopa is used as the substrate instead of tyrosine there is no induction period. Although dopa is required to catalyze the enzymatic oxidation of tyrosine, dopa itself is rapidly oxidized. Hence the rate of tyrosine oxidation is dependent on the rate of dopa oxidation.

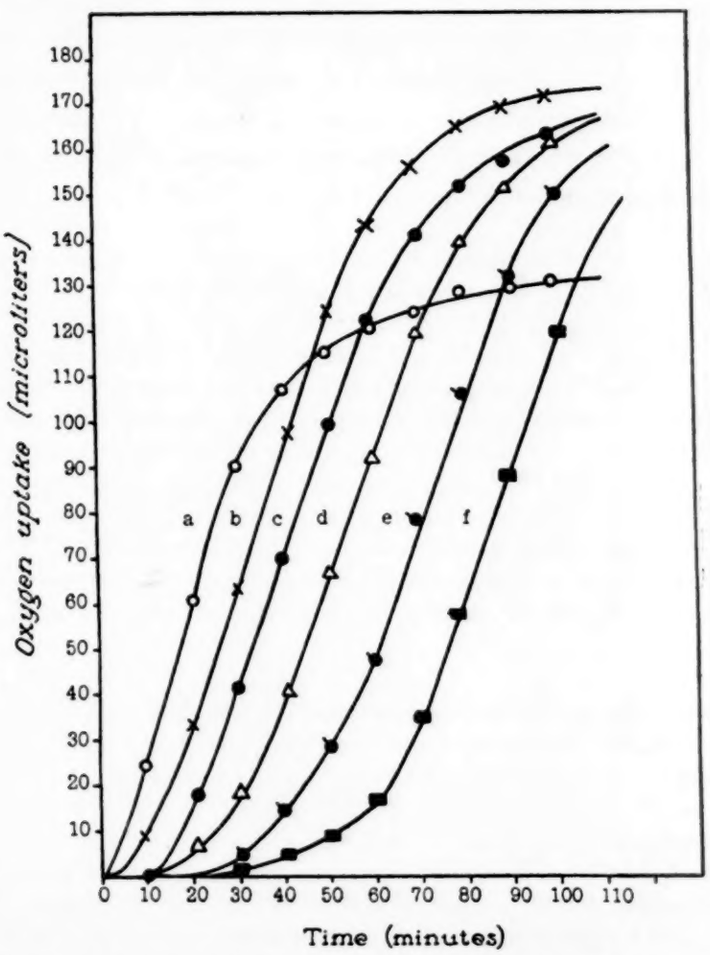


Fig. 2. EFFECT OF DOPA on induction period in enzymatic oxidation of tyrosine by mouse melanoma preparation at *p*H 6.8 and 38°C. *a* 0.5 mg. dopa; *b* 0.1 mg. dopa plus 0.4 mg. tyrosine; *c* 0.05 mg. dopa plus 0.45 mg. tyrosine; *d* 0.01 mg. dopa plus 0.49 mg. tyrosine; *e* 0.001 mg. dopa plus 0.50 mg. tyrosine; *f* 0.5 mg. tyrosine. (*J. Biol. Chem.* 178: 192, 1949).

It can be seen from figure 2 that after the induction period is over the rates of oxidation of tyrosine and dopa are practically identical. This observation at first glance seems difficult to understand, because one might expect that as the dopa formed from tyrosine is oxidized, the rate of reaction would diminish. The explanation of this finding is that dopa is not only oxidized in the reaction but also reformed during the oxidation, as discussed previously (fig. 1, lower part). Consequently, a significant amount of dopa is always available to shorten the induction period.

From what has been described it can be seen that the presence in the tyrosine-

tyrosinase reaction of any substance which is capable of reducing dopa-quinone back to dopa and thereby causes an accumulation of dopa should shorten the induction period. This is indeed the case. Either ascorbic acid or hydroquinone[5] shortens the induction period in tyrosine oxidation. Melanin, however, is not produced until all the ascorbic acid or hydroquinone has been oxidized. It is possible that the compounds related to dopa shorten the induction period by acting merely as nonspecific reducing agents which influence the induction period as described previously.

It should be pointed out that it is well known that *o*-dihydroxyphenyl compounds shorten the induction period in the oxidation of monophenols with tyrosinase obtained from plants and lower animals. Most of the work on this subject, however, has been done with catechol, phenol and *p*-cresol and not with dopa and tyrosine (54). No emphasis has been placed on structural specificity required by the orthodihydroxyphenyl compound. Actually, this structural specificity could play an important role in melanin formation in nature.

The mechanism by which substances such as dopa regulate the induction period of the tyrosine-tyrosinase reaction is not fully understood. Recently, the authors carried out potentiometric measurements using the platinum electrode on different solutions of tyrosine, dopa and tyrosinase obtained from the Harding-Passey melanoma (59). Solutions of tyrosine in 0.1 molar potassium phosphate buffer were found to be at a much higher potential than similar equimolar solutions of dopa. On the addition of tyrosinase to the tyrosine solution the potential began to fall. When the potential had fallen to a value approximately equal to that of the buffered dopa solution, oxygen uptake commenced. No appreciable oxygen uptake could be detected before the potential fell. As the oxidation proceeded and the tyrosine was converted to melanin, the potential began to rise; and when the oxidation was complete, the original potential was re-established. When the tyrosinase was added to the dopa solutions, oxidation began immediately and the potential began to rise. At the end of the reaction the potential reached a value similar to that obtained in the tyrosine-tyrosinase system. Small amounts of dopa added to the tyrosine solutions brought about an immediate lowering of the redox potential.

If the redox potentials were to influence the induction period, one could predict that increasing the tyrosine concentration of a tyrosine-tyrosinase reaction mixture would prolong the induction period. This was found to be the case. The redox potential of a system is established by the ratio of the quantity of reduced form of a substance to the oxidized form. An increase in tyrosine concentration increases the concentration of the reduced form of the substrate.

While the redox potential of the tyrosinase system may play a role in regulating the induction period (as well as tyrosinase activity in general) it is not the only factor involved. Equimolar quantities of DL-dopa are not as effective as is L-dopa in shortening the induction period, but there is no reason to believe that DL-dopa should establish a redox potential different from that obtained with the same quantity of L-dopa. It appears that dopa is a fairly specific catalyst for the enzymatic oxidation of tyrosine.

Nature of Tyrosinase

Tyrosinase can be obtained from various sources simply by grinding the tissues (for example, potato, fungus, melanotic tumor, and so forth) with an aqueous solution and then centrifuging the mixture at low speeds. The supernatant usually contains the active enzyme (47). Further purification can often be obtained by using ordinary procedures for protein fractionation.

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[5] In addition to shortening the induction period by virtue of its reducing properties, hydroquinone also appears to inhibit the enzyme.

There are many qualitative differences among tyrosinases prepared from various sources, but they all have three characteristics in common: *1*) All catalyze the oxidation of tyrosine to melanin (presumably by the series of reactions shown in fig. 1); *2*) the enzymatic reaction with the monohydroxyphenyl compound is catalyzed by some *o*-dihydroxyphenyl compound (dopa, catechol etc.); *3*) copper is associated with the activity of the enzyme. The first two points have been discussed previously.

Role of copper. Copper has been reported to be an essential part of tyrosinase prepared from mammalian (60), plant (61, 62) and insect tissue (63). Mouse melanoma tyrosinase can be inhibited by reagents that combine with copper (diethyldithiocarbamate, BAL, etc.) (60). This inhibition was reversed by the addition of an excess of cupric ions. Treatment of the enzyme with cyanide followed by dialysis resulted in a decrease of the copper content of the enzyme preparation and a loss of enzymatic activity. Addition of sufficient cupric ions resulted in almost complete restoration of activity. Other metals (iron, cobalt, nickel, magnesium, manganese and zinc) were ineffective in restoring enzymatic activity. Previous experiments by Kubowitz (61, 62) with plant tyrosinase and by Allen and Bodine (63) on grasshopper tyrosinase showed essentially the same results as those obtained with mammalian tyrosinase.

Properties of tyrosinase from different sources. As mentioned previously, there are several properties by which tyrosinase from different sources varies. Tyrosinase prepared from plant tissue can usually be obtained in colloidal solution. Tyrosinase obtained from mammalian tissue however is retained on ultramicroscopic cytoplasmic particles. As yet no method has been found by which the active enzyme can be separated from the particles. Hence, it must be realized that, when working with aqueous mammalian tyrosinase preparations, one is dealing with a suspension of particles which have molecular weights greater than those of most proteins.

Tyrosinase from plants and lower animals appears to be less specific in its action than is mammalian tyrosinase. Some plant tyrosinases are able to catalyze the oxidation of many phenol derivatives and orthodihydroxyphenyl compounds at a greater rate than the oxidation of tyrosine and dopa. With mammalian tyrosinase, on the other hand, tyrosine and dopa are oxidized at a much greater rate than any other substance related structurally to these amino acids (57, 58).

Sizer (64, 65), working with mushroom tyrosinase, reported some interesting findings on the oxidation of tyrosine present in the peptide chain of proteins. These findings support the contention that plant tyrosinase acts on combined as well as on free tyrosine. The effect of mammalian tyrosinase on tyrosine in proteins has not been tested; but, since this enzyme cannot catalyze the oxidation of tyrosine in which a hydrogen atom of the amino group is replaced by an acetyl or formyl group (57, 58), it is unlikely that tyrosine which is linked to another amino acid through its amino group could be oxidized by the mammalian enzyme.

Tyrosinase obtained from grasshopper eggs (66–69) occurs as a *protyrosinase.* This enzyme, unlike mammalian tyrosinase, must first be activated before it can exert any catalytic action on tyrosine (or related compounds). The activating factors are usually substances such as distilled water, sodium chloride, detergents, changes in *p*H or temperature.

Stability of tyrosinase. Tyrosinase from different sources varies greatly in its stability toward physical and chemical agents. The following information is available on the stability of a crude particulate suspension of tyrosinase from the Harding-Passey mouse melanoma (47). The enzyme preparations may be kept in solution at 5° C. for two months with no apparent loss of activity. Heating the preparations for ten minutes at 70° C. results in complete inactivation. Lyophilization does not alter enzymatic activity. Dialysis against water at 5° C. has no effect on the enzyme. Preparations may be kept at 5° C. in solutions ranging in *p*H from 4.7 to 8.0 for 24 hours without loss of enzymatic activity when reactions are later carried out at *p*H 6.8. Some fungus tyrosinases are inactivated after such treatment. The addition of 0.1 M acetate buffer at *p*H 4.7 to the mammalian enzyme preparation results in the formation of a precipitate which contains all the active material. The supernatant is inactive.

Plant tyrosinase is inactivated during reactions with various hydroxyphenyl compounds (54). The mouse melanoma tyrosinase referred to above does not appear to be readily inactivated during the reaction. If dopa is added to a reaction mixture which has previously oxidized dopa to melanin, the rate of oxidation is the same as that of the original reaction.

Effect of temperature on reaction rates. In general, the rate of enzymatic oxidation of tyrosine and dopa by preparations from the mouse melanoma increases with an increase in temperature. An increase in temperature also shortens the induction period in the oxidation of tyrosine. The temperature coefficient for the oxidation of dopa is only 1.2 at less than 37° C. (47). Above 37° C. the temperature coefficient increases to 1.7. This variation in the temperature coefficient is further indication that the oxidation of dopa to melanin is not a simple reaction. The biologic significance of the influence of temperature on reaction rates in melanin formation will be discussed later.

*Effect of p*H *on reaction rates.* It is difficult to evaluate the effect of *p*H on the enzymatic oxidation of dopa because dopa is readily oxidized in solutions kept at *p*H 7.0 or more even in the absence of the enzyme. With mouse melanoma tyrosinase the optimal *p*H for the oxidation of dopa is about 6.8. At *p*H 5.0 a marked decrease in the rate of oxidation occurs.

The induction period in the enzymatic oxidation of tyrosine appears to be at a minimum at *p*H 6.8. At *p*H values greater than and less than 6.8 the induction period increases. Above *p*H 8.5 and below *p*H 5.0 the induction period is prolonged indefinitely.

Effect of substrate concentration on total oxygen uptake. The total oxygen uptake during a reaction with tyrosinase is directly related to the initial concentration of tyrosine (or dopa) in the reaction mixture. If the concentration of substrate (within limits) is increased twofold, the total oxygen uptake is likewise increased twofold. The total amount of oxygen required to oxidize tyrosine and dopa to melanin is difficult to determine with great precision. Most reports (47, 70) indicate that each tyrosine and dopa molecule requires approximately five and four atoms of oxygen, respectively, for conversion to melanin. Variations in the concentration of enzyme affect somewhat the total amount of oxygen consumed in a reaction (71, 71a).

Is tyrosinase one or two enzymes? An important problem in the mechanism of melanin formation is whether tyrosinase is one or two enzymes. The following possibilities exist: *1*) One enzyme, tyrosinase, may be involved in melanin formation. If so, this enzyme possesses two distinct activities. First, it can effect the addition of an OH group to the benzene nucleus of a monohydroxyphenyl compound. Second, it can catalyze the removal of two hydrogen atoms from an *o*-dihydroxyphenyl compound. *2*) Two separate enzymes may be involved in melanin formation with each enzyme possessing a single activity. For example, one enzyme, tyrosinase, could catalyze the addition of an OH group to the benzene nucleus. A second and different enzyme, dopa-oxidase, could catalyze the removal of two hydrogen atoms from an *o*-dihydroxyphenyl compound. *3*) A third possibility to be considered is whether or not tyrosinase may be one enzyme plus an additional factor. These two substances together could possess the two catalytic activities described in the previous paragraphs.

Current evidence supports the view that tyrosinase is a single enzyme with two activities. This concept has been championed by Nelson and Dawson (54) and Mallette (72), whose notable work on plant and mushroom tyrosinase provided the experimental basis for the one-enzyme hypothesis. The following points lend support to this hypothesis: *1*) no tyrosinase preparation yet obtained has been satisfactorily demonstrated to catalyze the oxidation of monohydroxyphenyl compounds, but not that of *o*-dihydroxyphenyl compounds; *2*) the reverse statement is also true; namely, all enzyme preparations that catalyze the oxidation of *o*-dihydroxyphenyl compounds can catalyze the oxidation of monohydroxyphenyl compounds under the proper conditions; *3*) enzyme preparations have been obtained in which the ability to catalyze the oxidation of both the monohydroxyphenyl and *o*-dihydroxyphenyl compounds was proportional to the copper content of the preparation. Mallette and Dawson obtained from mushrooms a purified tyrosinase preparation which was homogeneous electrophoretically and almost homogeneous in the ultracentrifuge. The properties of this highly purified preparation were in accord with the foregoing points.

Recently a single-enzyme hypothesis was proposed to account for melanin formation in mammalian tissue (47). Studies with mammalian tyrosinase obtained from the Harding-Passey mouse melanoma showed that it was not possible to separate tyrosinase and dopa-oxidase activities although fractions with long induction periods in the oxidation of tyrosine could be obtained. These fractions were superficially free of tyrosinase activity; however, they catalyzed the oxidation of tyrosine rapidly and completely in the presence of small amounts of added dopa. For these reasons it was suggested that "the separate terms *tyrosinase* and *dopa-oxidase* be abandoned in favor of the single term *tyrosinase* to describe the enzyme (or enzyme complex) involved in the oxidation of both tyrosine and dopa to melanin." Further support of this concept is to be found in recent work which showed that N-acetyltyrosine and N-formyltyrosine are competitive inhibitors for the dopa-tyrosinase reaction (58). It is possible that dopa and the N-tyrosine derivatives compete for the same active centers on the enzyme molecule.

In the authors' opinion the term 'tyrosinase' is preferable to 'dopa-oxidase,' 'polyphenoloxidase' or 'phenolase.' The only justification for retaining the term dopa-oxidase (or 'dopase') is that

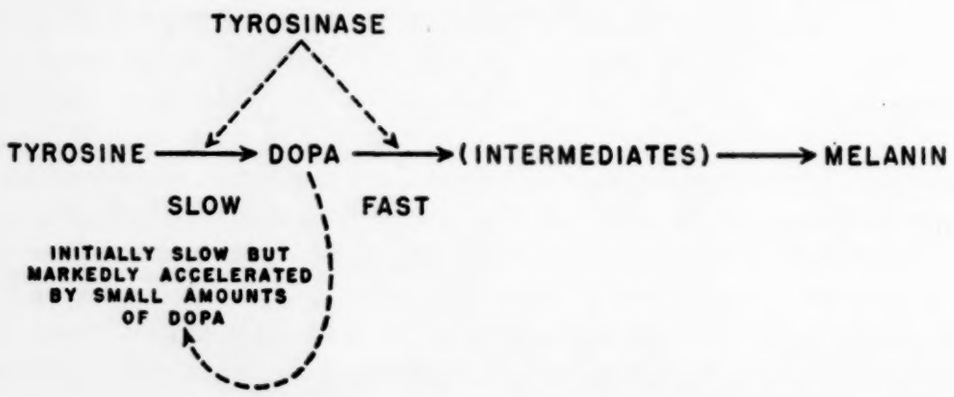
dopa, or some other dihydroxyphenyl compound, may be the initial substrate in the enzymatic formation of melanin because a dihydroxyphenyl compound is required to catalyze the tyrosine-tyrosinase reaction. Important objections to this view are, first, tyrosine is a more abundant natural substrate than dopa in mammalian melanin formation. Several dihydroxyphenyl compounds other than dopa can initiate the tyrosine-tyrosinase reaction; and it is possible that dopa comes, for the most part, only from the enzymatically oxidized tyrosine. Second, since dopa is readily oxidized to melanin in the absence of any specific enzyme, dopa-oxidase is often used as a non specific term. For example, the oxidation of dopa by oxidizing agents in an active cytochrome system is often mistakenly referred to as a dopa-oxidase reaction. For these reasons tyrosinase is a more suitable term.

Since no substance is more active or abundant than tyrosine as a substrate for the enzymatic formation of melanin by mammalian tissue, it is not desirable to use the general terms 'polyphenoloxidase' or 'phenolase.' However, tyrosinase might be considered a type of polyphenoloxidase or phenolase.

The two-enzyme hypothesis is not supported by direct experimental evidence. As yet, no enzyme capable of catalyzing the oxidation of tyrosine and dopa to melanin has been shown to be homogeneous by adequate critical experimental work. Until this is done, the possibility remains that the oxidation of tyrosine and dopa may involve separate enzymes.

The single-enzyme-plus-additional-factors hypothesis was suggested by Keilin and Mann (73). They prepared a purified oxidase from mushrooms, which they claimed was specific in catalyzing the oxidation of a small group of polyphenols. They expressed the belief that the oxidation of monophenols probably requires the presence of an additional factor. This view, with a change in emphasis, fits well with the one-enzyme hypothesis.

In accordance with the foregoing discussion and earlier statements it is suggested that the single term tyrosinase be used to include the separate terms tyrosinase and dopa-oxidase. This concept is illustrated diagrammatically below.



At the time Bloch carried out his important histochemical studies little was known about the optimal conditions for the enzymatic oxidation of tyrosine. This may account for the fact that Bloch, working with mammalian tissue slices, obtained melanin formation from dopa but not from tyrosine.

In some recent histochemical experiments (74) in collaboration with S. William Becker, Jr., we have demonstrated the formation of melanin from tyrosine in human

white skin which had been irradiated with ultraviolet radiant energy for one to five days before excision. Tissue slices cut from the biopsy material were incubated in tyrosine solutions at *p*H 7.1 for 24 to 48 hours. In paraffin sections of this material, there are large dendritic melanoblasts containing melanin granules in their cytoplasm, identical in their morphology with the 'dopa positive' cells obtained by Bloch. The catalytic effect of these cells on the oxidation of tyrosine to melanin is absent when the sections are heated for ten minutes at 100° C. Since tyrosine, in contrast to dopa which readily auto-oxidizes, is a stable amino acid which does not oxidize spontaneously to melanin *in vitro*, it is likely that the melanoblasts of human skin contain an intracellular oxidase, tyrosinase, similar to the enzyme described previously. The enzyme apparently exists in human skin in a partially inhibited state, and can be activated by ultraviolet radiant energy. The mechanism of this activation is not fully understood, but the inactivation of epidermal sulfhydryl groups by the ionizing radiation appears to play an important part.

NATURE OF MELANIN

The word 'melanin' is derived from the Greek *melas*, meaning black. It is used to denote various shades of brown and black pigments found in mammals, insects, plants and marine animals and produced *in vitro* by the oxidation of dihydroxyphenyl compounds. These pigments result from the polymerization of the oxidation products of dihydroxyphenyl compounds (dopa, epinephrine, catechol, and so forth) to relatively insoluble substances of high molecular weight. It is amazing that one term *melanin* has been used for many years to describe these natural and synthetic pigments even though there are a variety of melanins and even though no exact definition for the term can be given.

With the aid of the electron microscope Mason and co-workers (75) studied melanin granules from colored human skin; from the ciliary body, choroid, and iris of beef eyes; and from the S91 and Harding-Passey mouse melanomas. They found that the melanin granules were characteristically regular, spheroid particles. The particles appeared as formed elements and not simply as precipitated aggregates.

Because melanin is relatively insoluble in most solvents, it is difficult to isolate and purify from tissue sources. An additional factor making purification difficult is that melanin is often bound to protein in the tissues. Gortner described a melanoprotein present in sheep wool (76), and Greenstein and co-workers (77) have recently described a melanin-containing pseudoglobulin present in the S91 mouse melanoma. From the work of Sizer (64, 65) it appears that melanoproteins can be produced *in vitro* by the action of mushroom tyrosinase on the tyrosine within intact protein molecules.

An approximate composition of melanin from natural and synthetic sources, obtained by averaging the values in the literature, is as follows: carbon 57 per cent, hydrogen 3.5 per cent and nitrogen 9 per cent (35, 78). Oxygen is also present. Varying amounts of sulfur have been reported to occur in some natural melanins, but in some cases the sulfur can be removed (79). In other cases (77) the presence of sulfur seems to be associated with the amino acids of protein which is bound to melanin.

Melanin obtained from the ink sac of the squid or produced by the auto-oxidation of dopa or by the action of tyrosinase on tyrosine can be decolorized from jet black to light tan by sodium hydrosulfite (80, 81) or ascorbic acid (82). The tan-colored melanin produced with the former reducing agent can be changed to black again by the addition of potassium ferricyanide to the reaction mixture.[6] As a result of these findings, Figge (81) suggested that melanin can form a reversible oxidation-reduction system. These interesting findings must be explored further to determine the mechanism of

$$\text{Reduced Melanin (tan)} \rightleftharpoons \text{Oxidized Melanin (black)}$$

the process and to relate them to biologic systems. It is possible to predict that if there is reduction of melanin *in vivo*, the reduction does not occur at the site of melanin formation at the same time that melanin is being produced.

In 1939, Edwards and Duntley (83) suggested that the term melanoid be used when one is referring to the diffuse melanin pigmentation in the stratum corneum of human skin. They claimed that the corneum from the heel pad of a cadaver had a light-absorption maximum in the visible violet instead of the ultraviolet as does melanin. From this they concluded that the yellow pigment of the heel pad resulted from a disintegration of melanin. More work is needed to establish this point and until this is done the term melanoid should be used only with qualifications.

The various races (for example, white, oriental and Negro) appear superficially to have differently colored skins. All investigative work on this subject however indicates that melanin is the main pigment of human skin, and that the variation in color of skin from different races is due to the variation in quantity of melanin only (84). The spectrophotometric analyses of human epidermis reported by Brunsting and Sheard (85) and by Edwards and Duntley (83) support this view.

There are several ways to classify melanins because the nature and the sources of these pigments vary. In most classifications melanin is divided into two groups: natural and synthetic (86). The natural melanins can be subclassified according to their biologic source, and the synthetic melanins can be subclassified according to their mode of formation.

INHIBITORS OF MELANIN FORMATION

From a consideration of the properties of the enzyme tyrosinase which takes part in melanogenesis, and with a knowledge of the mechanism of the reactions involved in pigment formation it is possible to anticipate methods of stopping the reaction at certain stages. A list of some of the known inhibitors of melanin formation is given in table 1.

Substances That Combine with Copper

Inhibition of tyrosinase results in a decrease in the rates of the tyrosine-tyrosinase and dopa-tyrosinase reactions. Such inhibition can usually be achieved *in vitro* by binding (or removing) the copper ions, which are necessary for tyrosinase action, with substances that form weakly dissociable complexes with copper. Common in-

[6] However, vigorous oxidation of the black melanin apparently produces a substance which is first red and then colorless.

hibitors are organic sulfur-containing compounds (60, 87–89), hydrogen sulfide (4), carbon monoxide (4, 44) and cyanide ions (4, 44). The organic sulfur-containing compounds which have been used are phenylthiourea, α-naphthylthiourea, diethyldithiocarbamate, 2,3-dithiopropanol, cysteine, glutathione, thiouracil, thiourea and phenylthiocarbamide.

TABLE 1. INHIBITORS OF MELANIN FORMATION IN VITRO

I. Substances that combine with copper	
A. Phenylthiourea[1]	pK[2] = 5.60
B. Diethyldithiocarbamate	pK = 4.00
C. 2,3-Dithiopropanol (BAL)	pK = 3.85
D. Cysteine	pK = 2.90
E. Glutathione	pK = 2.35
F. Thiouracil[1]	pK = 2.05
G. Thiourea	pK = 1.75
II. Competitive inhibitors	
A. N-Acetyltyrosine	pK = 3.45
B. N-Formyltyrosine	pK = 3.35
C. Fluorotyrosine	pK = 2.50
III. Substances or conditions that prolong the induction period of tyrosine oxidation	
A. 'Tween-20'	
B. Changes in *p*H	
IV. Substances that combine with o-dihydroxy groups(?)	
A. Sodium molybdate[1]	
V. Substances that combine with orthoquinones	
A. Aniline	
B. Aminotyrosine	
C. *p*-Phenylenediamine	
VI. Reducing substances	
A. Ascorbic acid[1]	
VII. Hydroquinones	
A. Hydroquinone[1]	
B. *p*-Benzylhydroquinone[1]	

[1] Also inhibits melanin formation *in vivo*.

[2] pK is used to indicate the negative logarithm of the concentration of inhibitor that produces 50 per cent inhibition of the dopa-tyrosinase (Harding-Passey mouse melanoma) reaction.

Only four of these compounds (phenylthiourea, α-naphthylthiourea, thiouracil and phenylthiocarbamide) have been found to be effective *in vivo*. Phenylthiourea, α-naphthylthiourea or phenylthiocarbamide when administrated to black rats produces depigmentation (90, 91).[7] Removal of these substances from the diet results in a return of pigmentation. Thiouracil given to a patient with generalized melanosarcoma and melanuria has been shown to change the color of the urine from black to normal color (92). In a Negro patient under treatment with thiouracil for hyperthyroidism, areas of depigmentation developed (93).

Competitive Inhibitors

Some derivatives of tyrosine (for example, N-acetyltyrosine, N-formyltyrosine and 3-fluorotyrosine) are effective inhibitors of the tyrosine-tyrosinase and dopa-tyrosinase reactions (57, 58). These substances appear to act as competitive substrates by competing with the natural substrates, tyrosine and dopa, for active centers on tyrosinase. This inhibition has been observed *in vitro* and has not been studied *in vivo*.

[7] When the coat color of animals is considered (for example, in the black rat) the term depigmentation should not be taken to mean that existing melanin pigment in grown fur is decolorized. The term is used only to indicate that new hair does not contain normal amounts of melanin.

Substances That Prolong the Induction Period

Since there is an induction period in the tyrosine-tyrosinase reaction, a delay in the formation of melanin will occur if the induction period is prolonged. In this sense, substances or factors that lengthen the induction period may be considered to be inhibitors of pigment production. The detergent 'Tween-20' prolongs the induction period of the tyrosine-tyrosinase reaction but has no effect on the dopa-tyrosinase reaction (94). If the *p*H of a tyrosinase reaction mixture is increased to more than 7.5 or decreased to less than 6.5 the induction period is markedly increased. No adequate explanation of this phenomenon is known at present. Although to our knowledge, no clear-cut animal experiment or clinical observation has been shown to demonstrate an action of these inhibitors *in vivo* it is possible that examples may be found with further investigation.

Substances That Combine with Orthodihydroxy Groups

Sodium molybdate, when fed to cattle, causes a loss of coat color (95, 96). Since molybdate ions are known to combine with *o*-dihydroxy groups, a possible mode of action is by combination of the molybdate ions with compounds such as dopa and interference with their further metabolism (97). This view implies that in the presence of molybdate ions tyrosine can be oxidized to dopa but that dopa cannot be oxidized because it combines with molybdate ions. However, the fact that copper sulfate restores the coat color when given to molybdate-treated cows suggests that inhibition of pigmentation by direct combination of dopa with molybdate may not be an important factor. Molybdate may interfere with the absorption or utilization of copper, or with both.

Substances That Combine with Orthoquinones

In the usual process of melanin formation dopa is oxidized to dopa-quinone and the dopa-quinone is further oxidized. If dopa-quinone is removed from the reaction, melanin production will be inhibited. Aminophenyl compounds such as aniline, 3-amino-tyrosine, and *p*-phenylenediamine combine with orthoquinones and are inhibitors of melanin formation (53, 58). Action of these inhibitors *in vivo* has not been reported.

Reducing Substances

The *o*-quinones can be removed not only by the action of aminophenyl compounds but also by reduction to *o*-dihydroxyphenyl compounds by certain agents. In this way reducing substances can act as inhibitors of melanin pigmentation.

Ascorbic acid is a good example of this type of inhibitor. In the presence of ascorbic acid melanin cannot be formed by the action of tyrosinase on tyrosine or dopa until all the ascorbic acid is oxidized (98, 99). Large doses of ascorbic acid have been reported to decrease the pigmentation in patients with Addison's disease (100, 101, 82). A partial explanation of this phenomenon may be that excess ascorbic acid prevents melanin formation (see pages 106, 119 and 121, 122).[8]

[8] Ascorbic acid in large doses may also reduce the melanin in the skin to a relatively light-colored substance (82).

Hydroquinones

Compounds such as hydroquinone and *p*-benzylhydroquinone are effective inhibitors of melanin formation both *in vitro* and *in vivo*. Although their mode of action is not clear, they appear to act partly as reducing substances such as ascorbic acid. In addition these substances may act directly on tyrosinase.

Depigmentation in cats, rats and mice (102, 103) has been produced by adding hydroquinone to the diet. This effect of hydroquinone is reversible, since the animals become repigmented when hydroquinone is removed from the diet.

The *p*-benzylhydroquinone ('agerite alba'), which is used as an antioxidant in the processing of rubber, can produce depigmentation of human skin. The events leading to the discovery of this compound as the cause of occupational leukoderma in workers wearing rubber gloves containing agerite alba have been described by Oliver, Schwartz and Warren (104). Hydroquinone is reputed to have a similar but weaker action than does agerite alba. Perhaps the latter chemical is more effective because it is soluble and can penetrate through the skin more readily than can hydroquinone.

NUTRITIONAL FACTORS IN MELANIN FORMATION

It is well established that an abnormal increase or decrease in melanin pigmentation is associated with a variety of nutritional deficiencies. This phenomenon has been observed in several species of animals in addition to man. In some cases of abnormal pigmentation resulting from nutritional deficiency, it is difficult to determine the mechanism of the process. Most of the difficulty arises from the fact that several dietary factors are lacking in deficiency states, and usually it is not possible to relate the pigmentation directly to the lack of a single substance. Only a brief discussion of this interesting subject will be given here. A more detailed report of the literature can be found in a recent review by Frost (105).

Dietary Protein and Amino Acids

In 1923 Hartwell (106) reported that brown-black rats on a diet of bread, whole milk and vegetable kitchen scraps lost much of their color and became grey-fawn or even white. The animals became repigmented after 'food casein' was added to the diet. Hartwell suggested that the depigmentation resulted from a deficiency of tyrosine and tryptophane and showed that this deficiency could be corrected by feeding proteins such as 'food casein' which contain large amounts of these two amino acids. Since melanin is formed from tyrosine, any deficiency of tyrosine should result in a decrease of melanin production. Hartwell's findings can perhaps be partly explained on such a basis. However, the precise compositions of the diets used in her experiments were not reported, and it is not unlikely that a vitamin or mineral deficiency that was cured by 'food casein' also existed.

Rats on a synthetic diet poor in cystine and pantothenic acid have been found to become depigmented (107). Administration of cystine augmented the curative effect of calcium pantothenate. Lysine has been shown to be necessary for normal feather pigmentation in bronze poults (108). No adequate explanation of these findings is available.

It is unfortunate that more work on the amino acid requirements for normal pigmentation has not been carried out, especially with those amino acids that are necessary for the formation of tyrosinase itself.

Vitamins

Rats (108–113), dogs (112, 114), guinea pigs (112) and silver foxes (113) become depigmented when they are given synthetic diets deficient only in the filtrate factors of the vitamin B complex. These factors are not thiamine, riboflavin or pyridoxine and are not adsorbed on Fuller's earth. Pigmentation returns to normal when the animals are given adequate amounts of filtrate factors. In some instances, pantothenic acid, and, to a lesser extent, biotin have curative effects. In other cases pantothenic acid is not effective, but liver extracts containing relatively small amounts of pantothenic acid and yeast are curative. Frost and co-workers (115) suggested that a factor in liver and yeast potentiates the action of pure pantothenic acid. It remained for Wright and Welch (116) to indicate a possible interrelationship between pantothenic acid and pteroylglutamic acid. They showed that hepatic storage of pantothenic acid was increased after administration of folic acid concentrate and biotin to succinylsulfathiazole-fed rats in which depigmentation had developed. From these reports it appears that pantothenic acid is only one, although probably the most important, of the filtrate factors which act synergistically with pteroylglutamic acid in the development of normal pigmentation. Other filtrate factors that appear to play a role in melanogenesis are *p*-aminobenzoic acid and biotin.

In children on a multiple vitamin-deficient diet gray hair and depigmentation of the skin have been found to develop (117). After treatment with injectable liver extracts, powdered stomach and full diets, pigmentation gradually returns.

Definitive statements cannot be made concerning the mechanism by which vitamins of the B complex regulate melanin formation, as discussed previously. Studies *in vitro* of the effect of these factors on the enzymatic oxidation of tyrosine and dopa to melanin may clarify some aspects of this problem.

In contrast to the depigmentation associated with dietary deficiencies of some of the vitamins of the B complex, nicotinamide deficiency (pellagra) often results in increased pigmentation (118). Increased melanin pigmentation in patients with pellagra is seen most commonly at the site of the skin lesions. Pigmentation develops as the acute phase of the dermatitis subsides. The mechanism of this hyperpigmentation appears to be similar to that involved in postinflammatory pigmentation, namely, destruction of sulfhydryl groups during the acute dermatitis with a resulting increase in tyrosinase activity which persists until the concentration of the sulfhydryl group near the melanoblasts returns to normal. This mechanism will be discussed in greater detail in another section.

Increased pigmentation of the skin is also found in patients with sprue (119). The pigmentary signs usually resemble those found in cases of starvation (see later), but at times they may resemble those associated with Addison's disease.

An interesting type of hyperpigmentation is found in patients with vitamin A deficiency (120). The increased pigmentation in these patients is, for the main part, located at the sites of hyperkeratotic follicular papules which are present in this dis-

order. It is possible that the hyperpigmentation results from a decrease in the concentration of sulfhydryl groups in the skin. As will be seen in another section, sulfhydryl groups are *natural* inhibitors of tyrosinase because they combine with copper ions, which are necessary for tyrosinase action. Any reduction in the amount of substances that contain the sulfhydryl group, such as glutathione, near the site of melanin formation (melanoblast) represents a removal of a normal inhibitor of tyrosinase with a resulting increase in melanin production. Since vitamin A deficient patients usually have a diet inadequate in the sulfur-containing amino acids, and since such amino acids are used in keratin formation,[9] it is likely that those amino acids which are available form the protein of the hyperkeratotic papules at the expense of forming glutathione and other sulfhydryl compounds. It would be expected, as a result of this process, that the concentration of glutathione would be decreased in the vicinity of the melanoblast.

Usually little mention is made of pigmentation in cases of vitamin C deficiency, although a few reports (121, 122) indicate that this disorder may be associated with an increase in melanin pigment. In advanced cases of scurvy cutaneous hemorrhages occur, with a resulting increased deposition of hemoglobin breakdown products in the skin. Pigmentation, when it occurs, might result from a decrease in sulfhydryl groups in the skin which would follow an increase in the deposition of iron and copper compounds. This mechanism is similar to that suggested later in explanation of the increased melanin formation of hemochromatosis.

In conclusion, it can be stated that changes in melanin pigmentation are often seen in vitamin deficiency states. Decreased pigmentation is associated with inadequate intakes of the filtrate factors of the vitamin B complex. The mechanism of this process is not known. Increased pigmentation is found in deficiencies of nicotinamide and vitamins A and C and may result from a release of normal sulfhydryl inhibition of tyrosinase. The decrease in sulfhydryl groups can be produced in several ways.

Copper

Evidence from many different types of experimental work shows conclusively that copper is essential for normal pigmentation in mammals. Copper-deficient diets invariably result in depigmentation in rats (124, 125), cats (126), rabbits (126, 127) and cattle (128). Addition of trace amounts of copper salts to the deficient diet restores pigmentation. Other metals (iron, zinc and manganese) and vitamins of the B complex are ineffective by themselves in reversing the depigmentary process, although the administration of copper plus these substances is sometimes more effective than copper alone.

Further evidence for the necessity of copper in animal pigmentation is provided by the interesting reports on chronic molybdenum toxicity in cattle. Muir (95) first described the syndrome of depigmentation, intense diarrhea and emaciation in cattle which grazed on pastures (in Somerset, England) containing excessive amounts of

[9] It has been suggested (123) that keratin formation represents a normal 'excretory process' for glutathione elimination. According to this view sulfhydryl compounds such as glutathione are thought to be used as a source of cystine, which is necessary for keratin formation. The compounds are eliminated when keratin ceases to be active in metabolic processes.

molybdenum. The disease was cured by the administration of copper sulfate. This syndrome has been experimentally produced in cattle by prolonged feeding of molybdenum. At necropsy a decreased copper content of the liver has been found, indicating that excess molybdate in the diet interferes with copper metabolism.

Since copper is required for tyrosinase activity (60), it is reasonable to assume that a lack of dietary copper results in depigmentation because insufficient copper is available for the normal enzymatic formation of melanin.

Starvation

An unusual pigmentary disturbance of the skin in starvation recently has been described by European authors (129). A splotchy, dirty, grayish brown pigmentation appearing anywhere on the body but most often on the face was seen frequently at the end of World War II. This effect of starvation in humans is of special interest because the intake of several dietary factors such as vitamins, amino acids, fats and minerals is often reduced in a very low caloric diet.

In the latter part of 1944, a severe shortage of food and consequent starvation occurred in Western Holland (130). A dietary survey indicated that the average food consumed per person per day contained about 1000 calories. Since most of the food was obtained from vegetables, it is likely that the diet contained adequate amounts of most vitamins and copper, but inadequate amounts of many amino acids and fats, in addition to the low caloric intake. In these cases the same type of pigmentation developed as described in the preceding paragraph.

The situation in German concentration camps was much more severe (93). The inmates not only had a caloric intake of less than 1000 calories, but also were supplied with inadequate amounts of organic and mineral factors. In these cases a generalized grayish brown pigmentation developed and often there was a melanosis resembling that noted in Addison's disease.

With only the information available at present, it is difficult to account for the increased pigmentation. The fact that much of the increased pigmentation of the group in Western Holland occurred on the exposed areas suggests that ultraviolet radiation may have been a factor. Because of the low dietary intake of sulfur-containing amino acids[10] a decrease in the amount of substances containing sulfhydryl groups, which normally inhibit pigmentation, might be found in the skin. Further speculation does not seem justified until more is known about other factors, especially endocrine. Adrenal insufficiency may have been present.

HORMONAL FACTORS IN MELANIN FORMATION

It is well known that endocrine factors play an important role in melanin pigmentation in man and lower animals. Hence, it is surprising that although many experimental and clinical data have been obtained on this subject from studies on humans and lower species, practically nothing is available from experiments *in vitro*.[11] Nearly all the investigations in this field have been carried out upon intact

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[10] It appears likely that the relative concentration of sulfur-containing amino acids to phenylalanine and tyrosine (which are required for melanin formation) was reduced, because many vegetables have small amounts of cystine and methionine but large amounts of phenylalanine and tyrosine.

[11] This phrase 'experiments *in vitro*' is used here to mean only investigations carried out with isolated enzyme systems. Tissue culture experiments are included with findings *in vivo*.

organisms. In the present discussion efforts will be made to report and correlate information gained from observations on man and lower animals. In addition, the only two reports that we are aware of on studies with isolated enzyme preparations will be carefully evaluated.

Observations on Human Subjects and Experimental Animals

Sex hormones. Estrogens given orally have been shown to induce pigmentation in the nipples and areolae, and along the linea alba in humans (131). Parenteral administration of estrogens to guinea pigs induces pigmentation in the nipples and areolae (131). Particularly interesting is the development of pigmentation of the nipples following local unilateral application of estrogens to guinea pigs (131). This observation suggests that estrogens have a local pigmentogenic action on the melanoblast. The hyperpigmentation observed frequently in the nipples, areolae, linea alba and face during pregnancy has not been satisfactorily explained, but it is believed to be related to the high estrogen levels during gestation. The lack of pigmentation following administration of large doses of estrogens to women in the menopause is considered by Davis and co-workers (131) to be due to anatomic and functional changes in the pituitary occurring in the processes of aging, which interfere with the development of hyperpigmentation.

Testosterone induces melanin pigmentation when applied locally to sparrows' bills (132) and to the scrotum of the ground squirrel (133). Injection of androgens also increases melanin pigmentation in human male castrates (134). It has been observed that in male castrates little increased pigmentation develops upon exposure to ultraviolet light (134). However, if these men are given testosterone propionate several days after exposure to ultraviolet radiation, hyperpigmentation develops over the exposed areas.

Forbes (135) has reported darkening of the hair in male and female rats after implantation of both androgenic and estrogenic hormones in the form of pellets. Hamilton (136) working with tissue cultures of skin ectoderm from fowls found that androgens and estrogens accelerated the differentiation of melanophores.

As mentioned previously, estrogens and androgens appear to increase melanin pigmentation by acting directly on the melanoblasts. Their exact mode of action is obscure, and speculation on this subject is not justified at the present time.

Pituitary hormones. Cold-blooded vertebrates show striking pigmentary responses to changes in illumination, temperature and other factors (137). These alterations in pigmentation result from the expansion and contraction of dermal and epidermal melanophores, regulated by nervous and humoral influences. The humoral control is due to a blood-circulated pituitary melanophore hormone (or hormones). Injection of melanophore hormones into hypophysectomized frogs causes expansion of the cutaneous melanophores. Pituitary extracts from vertebrates of several classes, including mammals, contain melanophore hormones. It is interesting that although mammals do not have cutaneous melanophores, a rich store of melanophore-expanding hormone (intermedin) exists in the pituitary gland. Dawes (138, 139) has provided evidence that when amphibians with active melanophores are maintained for prolonged periods on illuminated black backgrounds, an absolute increase in amount or darkening in color of melanin results.

Clinical evidence indicates that the pituitary gland has some control of pigmentation in man. Patients with hypopituitarism often exhibit decreased melanin pigmentation. It is not known whether the pituitary gland exerts a direct or an indirect effect on pigmentation.

Adrenal hormones. Hyperpigmentation in animals following adrenalectomy has been observed by Ralli and Graeff (140, 141) and by Butcher (142). The former investigators produced nutritional achromotrichia in rats on a diet deficient in the filtrate factor and noted that adrenalectomy resulted in repigmentation of the hair. This repigmentation could be prevented by giving the animals desoxycorticosterone. In humans, also, total removal of the adrenal cortical tissue by surgery produces the clinical picture of Addison's disease with deep hyperpigmentation (143). These facts suggest that adrenal hormones under certain conditions have an inhibitory effect on melanin pigmentation. Hamilton (136) has demonstrated an inhibitory action of desoxycorticosterone on the development of melanophores in explants of skin from chick embryos grown in tissue culture. A recent report by Whitaker and Baker (144) showed the inhibitory effect of locally applied 11-dehydro-17-hydroxycorticosterone on melanin pigmentation and hair growth in black-hooded rats. In spite of these experimental findings, the exact mechanism of increased melanin pigmentation in adrenal hypofunction (Addison's disease) is not yet satisfactorily explained. The hyperpigmentation in this syndrome only rarely decreases and most often remains unchanged after replacement therapy with either adrenal cortical extracts or synthetic hormones such as desoxycorticosterone or 11-dehydro-17-hydroxycorticosterone. It is possible that the adrenal fraction responsible for the inhibitory effect of the adrenal gland on pigmentation either had been destroyed in preparation of the extracts or was present in insufficient quantity.

The slowly developing diffuse hyperpigmentation frequently associated with Addison's disease usually affects the parts of the body that are normally hyperpigmented, such as the axillae, areolae and anogenital regions. Exposed parts of the body such as the face and parts subjected to mechanical irritation also show increased pigmentation. The hair frequently darkens. Pigment normally present in the oral mucosa is increased in many patients. It is interesting that, although patients with Addison's disease have a generalized tendency toward hyperpigmentation, this abnormally increased deposition of pigment occurs in regions of the body most favored for normal melanin formation.

Areas of skin and mucous membrane, such as the axilla, groin, skin folds and oral cavity, which are at higher temperature than other regions, tend to be hyperpigmented. This might be associated with the fact that the rate of enzymatic oxidation of tyrosine to melanin like most enzyme reactions is dependent on temperature. The reaction is much more rapid at temperatures greater than 30° C. This subject will be discussed in more detail later in the paper.

Other hyperpigmented parts, such as the face and the dorsum of the hands, are exposed to light, which is known to stimulate melanin formation.

Much speculation has arisen concerning the mechanism of the processes which predispose to hyperpigmentation in patients with Addison's disease. According to

one of the oldest, and discarded, hypotheses, it is assumed that inhibition of the sympathetic nervous system results in increased melanin formation and that normally the adrenal gland stimulates the sympathetic system (145). Adrenal insufficiency would then predispose one to increased pigmentation due to lack of adrenal stimulation.

Bloch and Löffler (146) supported the view that epinephrine and melanin are derived from the same precursor and that if the adrenal glands do not utilize this substance for synthesis of epinephrine, it accumulates and is converted to melanin with subsequent melanosis.

Another hypothesis was proposed when it was found that ascorbic acid, normally present in high concentration in the adrenal gland, is decreased in Addison's disease presumably because of the destruction of adrenal tissue. Ascorbic acid is assumed to be a normal inhibitor of melanin formation, and any decrease in ascorbic acid in the tissues would result in hyperpigmentation (147). There is good evidence that this hypothesis is not correct, for if ascorbic acid normally inhibits melanin formation, dopa should accumulate in the serum. This has not been found to be the case (99).

Calkins (94) has suggested that the pituitary may have a primary role in human melanogenesis and that there may exist a pituitary-adrenal interrelationship (similar to the pituitary-thyroid axis) whereby adrenal hormones inhibit the release of intermedin (pituitary melanophore hormone). Primary adrenal cortical insufficiency would result in a compensatory overactivity of the pituitary with an increased output of intermedin followed by increased melanin synthesis. This hypothesis is interesting in view of some recent evidence (148) demonstrating an increased adrenocorticotrophic activity in the blood of patients with adrenal cortical insufficiency (Addison's disease). It is a well-known clinical observation that patients with secondary adrenal cortical insufficiency as a result of hypopituitarism (Simmonds' disease) only rarely develop hyperpigmentation.

An interesting observation,[12] which may indicate a possible role of the pituitary in human melanogenesis, has been made in a patient receiving adrenocorticotrophin (ACTH) in the arthritic service of the Mayo Clinic. This patient, a white male, developed marked pigmentation of the palmar creases, axillae, dorsum of the hands and feet, and in a recent operative scar. There were no indications of adrenal insufficiency which might account for the development of the pigmentation. The ACTH preparation, however, was found to contain appreciable melanophore hormone (intermedin) on bioassay in hypophysectomized frogs, a finding which may be relevant to the development of pigmentation under chronic treatment with pituitary preparations.

The authors would like to suggest that in Addison's disease there may be a decrease in concentration of sulfhydryl groups at the site of melanin formation. A decrease in sulfhydryl compounds in the blood of patients with Addison's disease has been reported (149). This observation indicates that the adrenal glands may

[12] For permission to cite the assay results and the clinical observations on this patient, the authors are indebted to Drs. A. Albert, R. G. Sprague and H. F. Polley, Mayo Clinic, Rochester, Minnesota.

play a role in regulating the metabolism of sulfhydryl compounds. As stated previously a decrease in glutathione or similar substances in the skin would tend to increase melanin pigmentation.

Several variations of the foregoing hypotheses can be found. It remains to be seen which if any of these views will receive experimental support.

Enzyme Studies

In 1940 Fostvedt (150) reported results of detailed experiments concerning the influence of melanophore hormones on the tyrosine-tyrosinase reaction. He worked with mealworm tyrosinase and melanophore hormone preparations obtained from the pituitary glands of different species of land and marine animals. Some hormone preparations apparently accelerated the tyrosine-tyrosinase reaction, as determined by oxygen uptake measurements, whereas others inhibited it. None of the preparations, however, had a notably marked effect on the tyrosine-tyrosinase reaction.

Fostvedt's systems had only weak tyrosinase activity, and usually no effect was noticeable for one or two hours. He reported differences between the experimental and control studies after this time, experiments sometimes being carried out for six hours. However, throughout the experiment the slopes of the oxidation curves for the test and control runs did not differ greatly, and it is difficult to ascertain whether an accelerating or inhibiting effect was present. Experiments should be done in which the tyrosinase activity is varied and the hormone concentration is kept constant, and vice versa. Also, since Fostvedt found relatively little leveling off of the oxidation curves even after 60 per cent of the tyrosine was oxidized, it would be interesting to prolong the experiments to determine whether or not all the oxygen consumed is used only to oxidize tyrosine to melanin. Fostvedt's experiments represent an interesting beginning in this field, but more work must be done before definite conclusions can be reached.

Figge and Allen (151) reported in 1941 that the inhibition of melanin formation by glutathione was released by estrone. Working with crude potato tyrosinase preparations, they measured melanin production in the tyrosine-tyrosinase reaction by colorimetric means. Glutathione prevented the formation of melanin, and estrone (in equimolar quantities with respect to glutathione) reversed the inhibition. This finding, if correct, would be of immense significance and might account for some of the action of estrone on pigmentation *in vivo*. Unfortunately, Figge and Allen did not use adequate controls since they did not determine the action of their tyrosinase preparations on estrone itself. Additional experiments, including some with purified tyrosinase, must be carried out to determine the effect of estrone on the tyrosine-tyrosinase reaction. The crude enzyme preparation probably contained estrinase (152). It would be interesting to know whether an oxidation product of estrone released the glutathione inhibition of tyrosinase or whether estrone itself was oxidized to a colored product.

NEUROGENIC FACTORS IN MELANIN FORMATION

In addition to the influence of nutritional and hormonal factors on the biochemical reactions involved in melanin formation, certain neurogenic factors play im-

portant roles. Most of the information on the neurogenic controls of pigmentation has been obtained from clinical observations.

A neurogenic control of melanogenesis in human skin is suggested by some recent transplantation experiments reported by Haxthausen (152a). When normal skin was transplanted to an area of vitiligo, the graft was found in a few months to become depigmented. Conversely, when the vitiliginous skin was transplanted to an area of normal skin, the pathologic skin graft gradually repigmented.

The hyperpigmentation seen in patients with the rare disease, acanthosis nigricans, is similar to that observed in Addison's disease and often develops when a malignant lesion of the viscera, either by direct extension or through pressure, involves the celiac plexus or chromaffin system. Reports have been made of abnormal pigmentation associated with neurologic and psychiatric disturbances (153–155).

These and other findings indicate that abnormalities of the nervous system can result in disturbances in pigmentation. The mechanism of the neurogenic control is unknown. It is hoped that some aspects of this fascinating subject can be clarified in the near future.

BIOCHEMICAL BASIS FOR MELANIN FORMATION IN CLINICAL CONDITIONS

The elaboration of melanin pigment in the epidermal melanoblast ordinarily depends on the available concentration of three substances: *1*) the enzyme tyrosinase —a copper-protein complex attached to ultramicroscopic particles in the cytoplasm of the melanoblast; *2*) a suitable substrate—usually tyrosine or dopa; *3*) molecular oxygen. If any of these substances is absent, the formation of melanin is impaired. This conclusion is self-evident when one considers the reactions involved in the oxidation of tyrosine to melanin as shown in figure 1.

The following are some disorders that deviate from this rule because of additional factors. As discussed earlier, dihydroxyphenyl compounds such as dopa or epinephrine can be fairly rapidly oxidized to deeply pigmented substances by molecular oxygen under certain physiologic conditions even in the absence of tyrosinase. In alkaptonuria, a paradihydroxyphenyl compound, homogentisic acid, is present in large amounts in body tissues and fluids (156). The oxidation of this substance in urine exposed to air (and possibly also in body tissues) produces a black pigment. A second example is the spontaneous oxidation of 5,6-dihydroxyindole to melanin in the tissues of patients with metastatic melanoma and melanuria. This will be discussed later.

It has been reported that dopa can be oxidized to melanin ***in vitro*** in the presence of an active cytochrome C-cytochrome oxidase system in the absence of tyrosinase (157). There is no indication that such a system plays any role in normal melanin formation ***in vivo***. The dopa-tyrosinase reaction is extremely rapid, and since melanin is usually produced only in certain specific cells which contain tyrosinase, it is unlikely that a cytochrome system plays a direct role in melanin formation. When melanin is produced in tissues which do not themselves contain melanoblasts, as in cases of alkaptonuria with ochronosis and some cases of generalized melanosis, the possibility exists that the cytochrome systems play a more direct part. But even in these cases there is no need to postulate that the reactions would depend completely on these systems.

The reaction of the three basic substances, tyrosinase, substrate and molecular oxygen, is controlled by several physicochemical factors which determine the rate of melanin formation: *1*) a catalytic substance, usually dopa, which can accelerate the tyrosine-tyrosinase reaction; *2*) chemical groups which normally inhibit copper en-

zymes, for example, sulfhydryl groups, normally found in the epidermis; 3) physical and chemical factors such as temperature, hydrogen ion concentration and oxidation-reduction potentials. The quantity of melanin produced by the cell depends on the over-all balance of these different forces as illustrated in figure 3.

Many substances, including dihydroxyphenyl compounds and reducing agents, catalyze the tyrosine-tyrosinase reaction. Of these substances dopa is the most effective now known (57, 58). The mechanism of this catalytic process was discussed previously.

It was pointed out in an earlier section that several compounds inhibit tyrosinase. Some effective inhibitors are naturally occurring organic sulfur compounds, such as glutathione and cysteine. These substances inhibit the enzyme by binding copper which is necessary for tyrosinase action. Ginsburg (158) demonstrated the presence

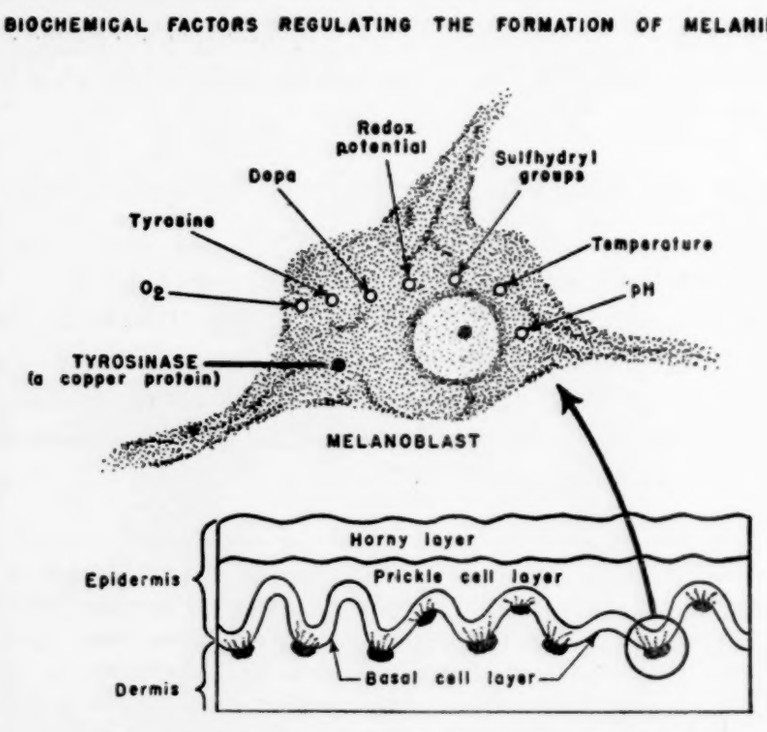

Fig. 3. FACTORS regulating the formation of melanin.

of sulfhydryl compounds in extracts of guinea pig skin, and recently Rothman and co-workers (29, 30) have found similar substances in isolated human epidermis. These findings suggest that sulfhydryl compounds occurring naturally in the skin may retain the enzyme tyrosinase in an inactive state by binding copper. Oxidation or inactivation of the sulfhydryl groups releases the bound copper, thus facilitating tyrosinase action.

From data already presented it can be seen that increasing the temperature, up to certain limits, of a tyrosine-tyrosinase reaction accelerates the reaction. The hydrogen ion concentration is also an important factor in melanin formation. The *p*H range 6.7 to 7.2 appears to be optimal. At higher values of *p*H the induction period in tyrosine oxidation is prolonged, and at lower values of *p*H tyrosinase activity is reduced. An additional factor is the oxidation-reduction potential of the system, which

may control the tyrosine induction period. High redox potentials are associated with long induction periods.

The state of oxidation of melanin itself may be important in the pigmentation of the skin. Melanin is light colored in the reduced form and black in the oxidized form. In the presence of reducing substances such as ascorbic acid, melanin is light colored (see page 106).

Most investigations of the enzymatic formation of melanin have been carried out with plant, insect and mammalian melanoma tyrosinase. Relatively little work has been done with tyrosinase from human epidermis (see pages 104, 105). For this reason only the factors which appear to be applicable to tyrosinase from all sources are discussed. It is assumed that the data apply to human epidermal tyrosinase as well. Justification for this assumption arises not only from the observation that some properties are common to all tyrosinases but also from the fact that tumors have not been found to contain enzymes differing from those present in normal tissues. There is no reason to believe that melanoma tyrosinase, which can easily be prepared, differs from normal skin tyrosinase, which can be isolated only in small quantities.

On the basis of the foregoing data attempts will be made to explain some normal and abnormal changes in pigmentation other than changes associated with nutritional, hormonal and neurogenic disturbances. It is not the purpose of this paper to review all known pigmentary disorders. Only conditions which illustrate biochemical regulatory factors will be discussed.

Suntanning

Ultraviolet irradiation appears to be concerned with melanin formation in at least four different ways, all of which tend to increase pigmentation. First, ultraviolet radiant energy catalyzes the oxidation of tyrosine to dopa (159). Small amounts of dopa thus formed can then catalyze the tyrosine-tyrosinase reaction. Second, the concentration of sulfhydryl groups in human epidermis is decreased after irradiation with ultraviolet light (160). Ionizing radiations (ultraviolet, roentgen, radium) produce an oxidizing agent (or agents) from water, which can oxidize sulfhydryl groups. In this process natural inhibitors of tyrosinase are removed. Third, the redox potential of human skin (161) decreases appreciably after irradiation. As stated previously, tyrosinase may be more active at relatively low potentials than at high ones. Fourth, cutaneous temperature is often but not always increased when one is exposed to ultraviolet irradiation, as in direct exposure to the sun on a warm, clear day. In addition, the erythema of the skin which results from exposure to ultraviolet light may elevate epidermal temperatures to values above normal. As already mentioned, increase in temperature accelerates melanin formation. The interrelationship of the various factors concerned in melanin formation by ultraviolet radiation are summarized in figure 4. In addition to its four probable effects on melanin formation, ultraviolet radiant energy also causes darkening (by oxidation) of melanin already present in the skin. A more detailed discussion of this interesting topic has recently been reported (162).

Heavy Metals and Melanosis

Increased melanin pigmentation is frequently observed when heavy metals such as arsenic, bismuth, iron, gold, silver[13] and mercury are deposited in the skin (163). Deposition of metals in the skin usually occurs when drugs containing heavy metals are used therapeutically. Patients with hemochromatosis have relatively large amounts of iron and copper deposited in the skin (164). The most reasonable explanation for these findings is that metals bind epidermal sulfhydryl groups and thereby release inhibition of tyrosinase. The increased tyrosinase activity results in increased melanin formation.

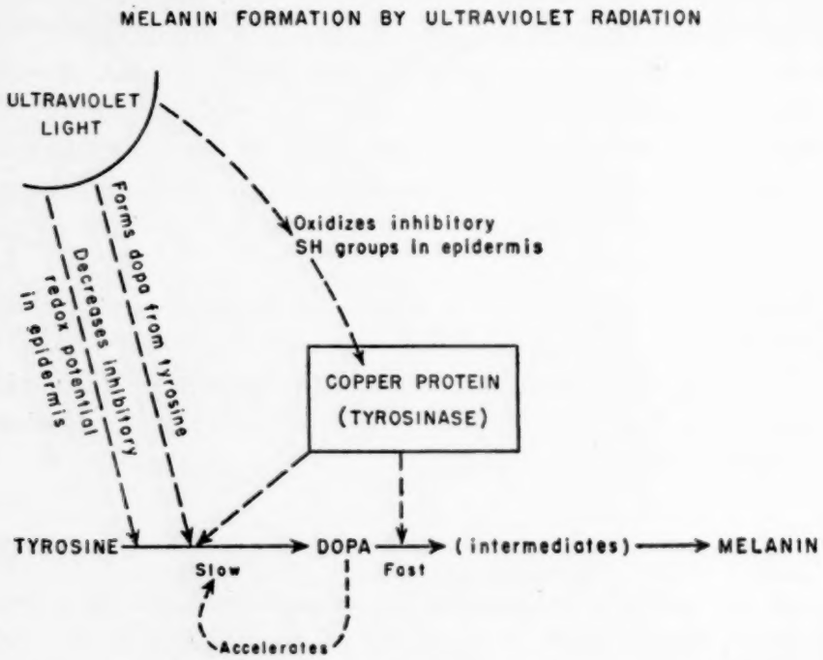

Fig. 4. FACTORS controlling the formation of melanin by ultraviolet radiant energy.

Heat and Pigmentation

Localized pigmentation often follows burns or chronic exposure to heat of limited areas of skin. The increased temperature may accelerate melanin pigmentation directly by accelerating the enzymatic oxidation of tyrosine and by increasing the rate of sulfhydryl group oxidation. After severe burns with destruction of melanoblasts, decreased pigmentation is noted. In these cases the enzyme tyrosinase is absent and melanin cannot be formed.

Postinflammatory Pigmentation

Increased pigmentation is seen in many patients who have had cutaneous inflammatory diseases. Rothman and co-workers (29) suggested that sulfhydryl compounds are oxidized or otherwise destroyed in some of the inflammatory processes with the result that melanin formation is increased.

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[13] The presence of silver in the skin (argyria) may be associated with only a slight increase in melanin pigmentation. Instead, there is often increased pigmentation due to the actual deposition of metallic silver. Small amounts of metallic silver may dissolve and be converted to silver ions. These ions could react with sulfhydryl groups to produce the slight increase in melanin.

Hyperthyroidism

Increased pigmentation has been noted in some patients with hyperthyroidism and decreased pigmentation in others (165). It is difficult at this time to interpret these conflicting observations. Hyperpigmentation might be related to the decrease in sulfhydryl compounds which has been reported in hyperthyroidism. Hypopigmentation might be due to decreased tyrosine concentration in the skin as a result of increased conversion of tyrosine to thyroxine and other substances due to the hypermetabolic state. The balance between the relative concentration of sulfhydryl compounds and tyrosine may determine the pigmentary changes in patients with hyperthyroidism.

Melanuria and Generalized Melanosis

It is not unusual to observe melanuria and rarely generalized melanosis in patients with metastatic melanoma (166–174). Linnell and Raper (175) showed that a simple derivative of 5,6-dihydroxyindole was present in the urine of a patient with a metastatic melanoma and melanuria. Melanotic tumors may produce such large amounts of the oxidation products of tyrosine that some of these compounds (dopa, 5,6-dihydroxyindole, etc.) are released into the general circulation before being completely oxidized to melanin in the tumors. These substances could be oxidized to melanin in tissues distant from the tumor site even in the absence of tyrosinase (fig. 1).

Phenylpyruvic Oligophrenia

Phenylpyruvic oligophrenia is a rare disease in children characterized by mental deficiency and increased urinary excretion of phenylpyruvic acid and phenylalanine (176). These patients characteristically have light skin, blond hair and blue eyes (177–179). They do not tan when exposed to sunlight. This condition is considered to be an inborn error of metabolism in which phenylalanine cannot be converted to tyrosine. It is possible that tyrosine, the substrate in the enzymatic formation of melanin, is present in amounts inadequate for melanin synthesis.

Normally, metabolic requirements for tyrosine are fulfilled directly from dietary sources and from oxidation of phenylalanine. Patients with phenylpyruvic oligophrenia obtain tyrosine only from the diet, and they have reduced amounts of tyrosine in the blood. It is possible that the available tyrosine is utilized for the production of essential hormones, (for example, thyroxine) and proteins rather than for conversion to melanin.

Albinism

Partial or complete absence of melanin has been found to occur as a recessive trait in all mammals that have been studied. The inability of albinos to form melanin results from absence of melanoblasts in the epidermis. Since tyrosinase is contained within the melanoblast, the substrate tyrosine, although present in adequate amounts, is not catalytically oxidized to melanin.

Normal Variations in Pigmentation

A question often asked is why certain individuals are more pigmented than others. In normal individuals, pigmentation varies from very light to near black. Among

the factors controlling melanin formation, shown in figure 3, the tyrosine, dopa and oxygen concentrations and the temperature and *p*H would be expected to be approximately the same for all healthy people. The concentrations of tyrosinase and sulfhydryl compounds (and possibly the redox potential) are likely to vary in different individuals and to depend in large part on hereditary factors. The state of oxidation of melanin itself may vary. It is not possible at this time to conclude which of these factors plays the dominant role in determining the pigmentation of an individual.

SUMMARY

In this review an endeavor has been made to describe the biochemical mechanisms of melanin formation and to correlate the enzymatic processes involved therein with experimental and clinical observations on melanin pigmentation.

The enzyme tyrosinase, a copper-protein complex, catalyzes the oxidation of tyrosine to dopa and the oxidation of dopa to melanin. Tyrosinase is widely distributed in nature and can be found in plant, insect, marine animal and mammalian tissues. In all these species tyrosinase plays an important role in melanin pigmentation.

Until recently it was believed that mammalian tissue did not contain tyrosinase but contained instead, an enzyme called 'dopa-oxidase,' which supposedly catalyzed only the oxidation of dopa to melanin. It is now known that the original distinction between tyrosinase and dopa-oxidase is no longer valid. Hence, it is suggested that the single term tyrosinase should be used instead of the separate terms tyrosinase and dopa-oxidase.

While tyrosinase obtained from different species has some unique properties depending on the particular source, three characteristics are common to tyrosinase, under proper conditions, regardless of its origin: *1*) the oxidation of tyrosine to melanin is catalyzed by tyrosinase; *2*) the tyrosine-tyrosinase reaction is catalyzed by some *o*-dihydroxyphenyl compound, for example, dopa; *3*) the activity of the enzyme is associated with copper ions. Current investigations indicate that tyrosinase is a single enzyme, although unequivocal proof of this is lacking. The preparation and properties of tyrosinase are discussed.

Various substances inhibit melanin formation *in vitro* and *in vivo*. The mechanism of the inhibition depends on the particular step which is blocked of the tyrosinase-catalyzed series of reactions by which tyrosine is converted to dopa, and eventually to melanin. Melanin pigmentation in mammals is regulated by biochemical factors, some of which are well defined, such as the concentrations of enzyme, substrate, hydrogen ions, sulfhydryl groups, and so forth. Some of these factors, as well as others which are as yet unknown, are influenced by nutritional, hormonal and neurogenic control. The biochemical basis for melanin pigmentation in several clinical conditions has been presented.

We should like to express our indebtedness to Marguerite Rush Lerner for her invaluable assistance in the preparation of this review. We wish to acknowledge the contributions made by Dr. W. H. Summerson and Dr. E. Calkins, who aided in carrying out the experimental work which formed the basis for some of the concepts of the biochemistry of melanin formation presented in this paper. For their helpful criticisms and suggestions, we want to thank the following: Drs. L.

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